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The role of leaf hydraulic conductance dynamics on the timing of leaf senescence

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Abstract. We tested the hypothesis that an age-dependent reduction in leaf hydraulic conductance (K_{leaf}) influences the timing of leaf senescence via limitation of the stomatal aperture on xylem compound delivery to leaves of tomato (*Solanum lycopersicum* L.), the tropical trees *Anacardium excelsum* Kunth, *Pittoniotis trichantha* Griseb, and the temperate trees *Acer saccharum* Marsh. and *Quercus rubra* L. The onset of leaf senescence was preceded by a decline in K_{leaf} in tomato and the tropical trees, but not in the temperate trees. Age-dependent changes in K_{leaf} in tomato were driven by a reduction in leaf vein density without a proportional increase in the xylem hydraulic supply. A decline in stomatal conductance accompanied K_{leaf} reduction with age in tomato but not in tropical and temperate tree species. Experimental manipulations that reduce the flow of xylem-transported compounds into leaves with open stomata induced early leaf senescence in tomato and *A. excelsum*, but not in *P. trichantha*, *A. saccharum* and *Q. rubra* leaves. We propose that in tomato, a reduction in K_{leaf} limits the delivery of xylem-transported compounds into the leaves, thus making them vulnerable to senescence. In the tropical evergreen tree *A. excelsum*, xylem-transported compounds may play a role in signalling the timing of senescence but are not under leaf hydraulic regulation; leaf senescence in the deciduous trees *A. trichanta*, *A. saccharum* and *Q. rubra* is not influenced by leaf vascular transport.

Additional keywords: chlorophyll, leaf anatomy, leaf phenology, photosynthesis, tomato, vascular transport.

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Introduction

Leaf senescence is a key developmental transition in which chlorophyll, cytoplasmic proteins and cellular membranes are degraded, and nutrients are remobilised from senescing leaves to young leaves, seeds, fruits, storage tissues and new buds (Himelblau and Amasino 2001). It has been proposed that leaf senescence functions to optimise plant nitrogen distribution (Ono *et al*. 2001); to reduce water use, thus facilitating plant survival under drought conditions; and to boost plant fitness by accelerating the reproductive output in monocarpic plants under water stress (Munné-Bosch and Alegre 2004). Despite its importance, it is not known how plants determine the onset of leaf senescence (Lim *et al*. 2007). Studies in a tropical moist forest of Panama demonstrate that leaf fall is not determined by soil water availability, suggesting instead that vapour pressure deficit (VPD) or irradiance trigger leaf abscission (Wright and Cornejo 1990; Wright 1991; Wright and Vanschaik 1994). Temperature and photoperiod have been commonly identified as environmental drivers of leaf senescence in temperate forests but hypotheses to explain the underlying physiological mechanisms lack experimental evidence (Estrella and Menzel 2006).

The marked changes in expression of senescence-associated genes (SAGs) that accompany the onset of senescence in herbaceous plants indicate that it is a highly regulated developmental transition (Buchanan-Wollaston *et al*. 2003; Guo and Gan 2005; Lim *et al*. 2007). SAG expression is an integrated response of developmental age and environmental stressors such as UV-B, ozone, nutrient limitation, temperature, shading, pathogens and phytohormones (Lim *et al*. 2007). A decline in photosynthesis has been proposed as a signal for age-dependent leaf senescence (Hensel *et al*. 1993) but the evidence remains controversial (Guo and Gan 2005). In *Arabidopsis thaliana* (L.) Heynh. mutants and transgenic tobacco (*Nicotiana tabacum* L.) plants with reduced photosynthetic activity, age-dependent leaf senescence is delayed compared with wild-type plants (Miller *et al*. 2000; Woo *et al*. 2002). Opposing hypotheses positing that sugar starvation or sugar accumulation in leaf cells initiate senescence lack decisive evidence (van Doorn 2008). To date, how the developmental age of leaves is recognised during senescence and the nature of the signal is poorly understood.

The plant vascular system, as the nexus of hormone and nutrient transport, is well positioned to act as an age-dependent signalling system controlling leaf senescence. It is sensitive to a wide range of physiological (water and nutrient availability), environmental (light and temperature) and age-related factors identified as inducing leaf senescence (Sack and

Holbrook 2006; Lim *et al*. 2007). An age-dependent decline in the conductance of the liquid phase of water through leaves (leaf hydraulic conductance (K_{leaf}); mmol m^{-2} s^{-1} MPa^{-1}) was associated with the onset of leaf fall (Salleo *et al*. 2002; Brodribb and Holbrook 2003*a*; Lo Gullo *et al*. 2004) but a causal role for K_{leaf} in leaf senescence was not proposed. Because K_{leaf} is often a major bottleneck in the movement of water through plants (Sack and Holbrook 2006), it is the most likely component of the vascular system to act as a hydraulic regulator of leaf senescence. Through its effect on leaf water status, K_{leaf} can limit stomatal aperture (Meinzer 2002; Brodribb and Holbrook 2003*b*), thus influencing the delivery of key xylem-transported regulators of leaf senescence.

Both cytokinins and nitrogen compounds are transported by the xylem and have been identified to play a role in leaf senescence (Pourtau *et al*. 2004; Boonman *et al*. 2007). Cytokinins are well known to delay leaf senescence and even cause regreening of yellowing leaves of species such as *A. thaliana*, tomato (*Solanum lycopersicum* L.) and tobacco (Richmond and Lang 1957; Gan and Amasino 1995; Gan and Amasino 1996; Lo Gullo *et al*. 2004; Guo and Gan 2005). In *A. thaliana*, experimental manipulations that reduce transpiration but not stomatal conductance induce senescence, with decreased cytokinin delivery implicated as the driver of leaf chlorophyll degradation (Boonman *et al*. 2007). Soil nitrogen deficiency in herbaceous species leads to sugar accumulation in leaves (Ono and Watanabe 1997), which, in turn, triggers the expression of SAGs (Rolland *et al*. 2006). The highly specific *SAG12* encoding a cysteine protease can be induced by 100-fold during leaf senescence by growing *A. thaliana* plants in 2% glucose in combination with low nitrogen availability (Pourtau *et al*. 2004; Wingler *et al*. 2004).

K_{leaf} reduction with leaf age has been reported in tropical and temperate trees (Salleo *et al*. 2002; Brodribb and Holbrook 2003*a*) and herbaceous plants (Lo Gullo *et al*. 2004), although whether this is due to changes in the leaf xylem or mesophyll hydraulic pathways remains unclear. A possible explanation for K_{leaf} reduction with leaf age is that the xylem's structural components deteriorate, thus impairing leaf function. For example, degradation of pit membranes can make aging vessels more prone to air-seeded embolisms by lowering the pressure threshold required for embolism formation (Sperry *et al*. 1991). Alternatively, the reduction of K_{leaf} with age could take place in the extra-xylary pathways, where water may encounter most of the hydraulic resistance within leaves (Nardini and Salleo 2005; Brodribb *et al*. 2007). Changes in protoplast and parenchymal wall properties have been implicated in the ontogenetic reduction of mesophyll conductance (Aasamaa *et al*. 2005). Simple thickening of the cell walls in the apoplastic pathway or structural changes in the plasmodesmata through the symplast could significantly influence K_{leaf}. Age-related accumulation of the phloem's immobile compounds in leaf cell membranes such as calcium (McLaughlin and Wimmer 1999) may contribute to reduce cell membrane permeability via downregulation of aquaporin conductance (Kaldenhoff and Fischer 2006; Kaldenhoff *et al*. 2008).

In this study, we examined the hypothesis that a decline in K_{leaf} as leaves age influences the onset of leaf senescence via limitation of the stomatal aperture on xylem compound delivery to the leaves of tomato plants, and tropical and temperate tree species. We first conducted a comprehensive study of greenhouse tomato plants to test our hypothesis under controlled laboratory conditions. We then scaled up our experiments to more challenging field conditions in the canopy of tropical and temperate trees. To test our hypothesis we determined (1) whether the K_{leaf} decline precedes a significant reduction in leaf chlorophyll content at the onset of leaf senescence; (2) if the K_{leaf} drop limits gas exchange, thus impacting leaf xylem compound delivery into leaves; and (3) whether downregulating the flow of xylem-transported compounds into leaves, through a reduction in transpiration rates with open stomata, induces earlier leaf senescence.

Materials and methods

Plant material

Wild-type glasshouse tomato plants (*Solanum lycopersicum* L. var. MP-1) were sampled when plants were 2–3 months old and had at least 16 nodes. Tomato plants were grown in 4-L pots with a soil mixture of Fafard Mix#3B (Sungro, Agawan, MA, USA). Soil was irrigated to field capacity twice a day at 0800 hours and 1400 hours. Plants were maintained at glasshouse conditions of 25°C day : 19°C night during a 13-h photoperiod. A set of five plants was used to determine the relationship between average leaf age and position (leaf node from apex, LNA), where leaf age (days) = $2.33 \times LNA - 2.94$ ($R^2 = 0.9694$, $P < 0.001$; $n = 5$). In tropical and temperate trees, several leaves per individual were measured monthly from expanded to presenesced leaves on randomly selected sun branches. Three tropical canopy trees of *Anacardium excelsum* Kunth and *Pittoniotis trichantha* Griseb up to 45 m in height were accessed using the Smithsonian Tropical Research Institute crane located in Parque Nacional Metropolitano, Panama. Young leaves were fully expanded and labelled at the beginning of the dry season in November 2008. Field experiments in temperate trees were performed at Harvard Forest, Petersham, Massachusetts, during the summers of 2008 and 2009 on three individuals each of the deciduous tree species *Acer saccharum* Marsh. (sugar maple) and *Quercus rubra* L. (red oak). Individuals were distributed along a road near the main laboratory facilities, and were accessed with a ladder and the Harvard Forest canopy lift. Young leaves completed expansion in mid- to late June, and were shed in September and October in *A. saccharum* and *Q. rubra*, respectively. Leaf age was calculated as the number of days from the date of completed leaf expansion.

Leaf chlorophyll content and senescence

A significant decline in leaf chlorophyll content was used as an indicator of the onset of leaf senescence (Fig. S1, available as Supplementary Material to this paper). Chlorophyll degradation has been reported to precede the remobilisation of nitrogen from chlorophyll-binding proteins (Hörtensteiner 2006) and dismantling of thylakoid membrane proteins (Thomas *et al*. 2002). The expression of the senescence-specific gene *SAG12* in tomato leaves has been associated with a significantly lower chlorophyll content compared with young leaves (Swartzberg *et al*. 2006). Leaf chlorophyll absorbance was measured *in situ* with a chlorophyll meter (SPAD 502, Minolta, Warrington, UK). To calibrate the SPAD readings, total chlorophyll content ($a + b$) was measured by spectrophotometry (Lee *et al*. 2003). Linear

regressions were used to convert SPAD chlorophyll absorbance measurements to chlorophyll $a+b$ content ($\mu g\,cm^{-2}$) (Fig. S1).

Leaf hydraulic conductance

Water flow through leaves was measured using a modified evaporative flux method (Sack *et al*. 2002; Brodribb and Holbrook 2006). Petioles of leaves were submerged and cut under water to prevent the formation of embolisms. The base of the petiole was immediately connected via silicone tubing to a flow meter consisting of polyether ether ketone (PEEK) tubing (McMaster-Carr, Robbinsville, NJ, USA) with a known conductance (K) in which the pressure drop (ΔP) was continuously monitored in LABVIEW (National Instruments, Austin, TX, USA) with a pressure transducer (PX26, Omega, Stamford, CT, USA). PEEK tubing flow (F) was calculated by an Ohm's law analogy as $F = K \times \Delta P$. Leaf flow was determined by applying the principle that two conductors connected in series have equal flows. Leaf transpiration was induced with a 500-W halogen lamp (Bayco, Wylie, TX, USA) providing a PAR of 900–1300 $\mu mol\,m^{-2}\,s^{-1}$ (measured using a LI-250 light meter (LI-COR, Lincoln, NE, USA). A cooling fan was used to reduce the leaf boundary layer, and a water bath was placed between the leaf and the halogen lamp to prevent overheating. Leaf temperatures measured with an infrared sensor (OS543, Omega) on three leaflets per leaf ranged from 26°C to 32°C with a maximum temperature difference between leaflets of 4°C. Ambient relative humidity near the leaf surface varied from 25% to 60% (SAM990DW digital sling psychrometer, McMaster-Carr). A steady flow was usually reached no earlier than 45 min after connecting the leaves to the water supply and exposing them to the environmental conditions generated by the fan and the halogen lamp. Some leaves were preadapted to these conditions in a replica set up to optimise the use of the flowmeter. Flow values were recorded when the variation was less than 5% of the mean. Leaf water potential was measured with a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Flows were normalised by leaf area determined with a LI-300 leaf area meter (LI-COR). K_{leaf}, as determined by the evaporative flux method, was calculated using Eqn 1:

$$K_{leaf} = \frac{flow}{water\ potential}. \quad (1)$$

Prior to measurements, the flowmeter and an intravenous bag that served as a water reservoir were bleached to prevent bacteria growth, rinsed and filled with deionised and degassed water filtered with a 0.2 µm mesh (Acrodisc syringe filter, Pall, Ann Arbor, MI, USA).

The hydraulic conductance of the most apical tomato leaflet ($K_{leaflet}$) was measured by the evaporative flux method as described above (Sack *et al*. 2002; Brodribb and Holbrook 2006) and leaf-specific rachis hydraulic conductance was determined with a flow meter (Brodribb and Holbrook 2006). The most basal segment of the rachis between the main plant axis and the first petiolule (hereafter referred to as the petiole) was excised underwater with a clean razor blade. All leaflets were excised with only the rachis remaining. Leaf-specific rachis hydraulic conductance was calculated as $K_{rachis} = flow \div driving\ pressure$, normalised by leaf area supplied by the rachis (measured using a LI-1300 meter (LI-COR)).

Tomato leaf anatomy

Vein density measurements were performed by sampling leaf discs 8 mm in diameter from the central portion of the terminal leaflet of each LNA. Procedures for clearing tissues were based on methods described by Berlyn and Miksche (1976) and Scoffoni and Sack (2010). Samples were imaged at 4× magnification on an Olympus BH2 (Olympus America Inc., Center Valley, PA, USA) equipped with an AxioCam HRc camera (Carl Zeiss MicroImaging, LLC, Thornwood, NY, USA). Images were analysed using ImageJ software (National Institute of Health, Bethesda, MD, USA) with vein density (2° and higher) calculated as total vein length per sample area ($mm\,mm^{-2}$).

Petiole xylem area was measured on cross-sections stained with a solution of 95% alcoholic phloroglucinol plus HCl. Petiole segments ~0.5 mm in length were cut near the most proximal leaflet then placed on a microtome (Reichert, Depew, NY, USA), submerged in embedding medium (OCT compound 4583, Tissue Tek, Sakura Finetek, Torrance, CA, USA), frozen and sectioned at 80 µm thickness. Sections were mounted on slides with phloroglucinol for 20 min before imaging at 3× using a digital camera (Axiocam HRc, Zeiss, Thornwood, NY, USA) attached to a dissecting scope (SZ60, Olympus). Petiole xylem area was manually selected from stained regions and area measured in ImageJ (National Institute of Health, Bethesda, MD, USA).

To determine the size distribution of conductive vessels in the petiole of young and old leaves, we performed leaf live staining with acid fuchsin. Petiole sections were observed using a compound microscope (BH-2, Olympus) and an image taken of the third xylem quadrant starting from the top of the petiole in a clockwise direction, with a digital camera (Axiocam HRc, Zeiss) at 10× magnification. Vessels were outlined in ImageJ (NIH) and vessel cell wall traces were measured for area, perimeter, and major and minor axis dimensions.

Leaf gas exchange

Maximum assimilation (A) and stomatal conductance (g_s) measurements were performed with a LI-6400 meter (LI-COR). Plants were measured on sunny mornings from 0900 hours to 1200 hours. Leaves of different ages were measured in a random order over the sampling interval to avoid any systematic effect associated with recording time. In the glasshouse, leaf chamber settings were light saturating levels of 1200 $\mu mol\,m^{-2}\,s^{-1}$, ambient relative humidity, a block temperature of 25°C, a flow rate of 500 $\mu mol\,s^{-1}$ and average CO_2 of 375 ± 5 mol $CO_2\,m^{-2}\,s^{-1}$. At the tropical site, leaf chamber PAR was set according to light curves, which demonstrated that 1800 $\mu mol\,m^{-2}\,s^{-1}$ with 10% blue light was saturating. Reference air CO_2 levels in the chamber were regulated with soda lime to 370 ± 5 parts per million. Relative humidity was allowed to vary in the natural range, block temperature was maintained at 30°C and flow rates were set at 500 $\mu mol\,s^{-1}$. At the temperate site, a LI-COR cuvette was set up at a saturating PAR of 1500 $\mu mol\,m^{-2}\,s^{-1}$, flow rates of 500 $\mu mol\,s^{-1}$, CO_2 levels of 380 ± 5 parts per million, a block

temperature of 27°C and ambient relative humidity. In each individual, three to four canopy sun leaves were randomly selected for gas exchange measurements. Values were taken after stomatal conductance reached stable values, usually no longer than 5 min after clamping the leaf on the chamber.

Manipulation of xylem delivery rates

We tested the hypothesis that a decline in K_{leaf} influences leaf senescence via its effect on the delivery of xylem-transported compounds by reducing leaf transpiration with a novel system of canopy humidity chambers (Fig. S2). Leaves were subject to reduced transpiration rates (but open stomata) by maintaining the chamber humidity at more than 90% during the daylight period (Fig. S3). Tomato leaves between the fourth and sixth nodes from the plant apex were selected for the high humidity treatment. The most proximal older leaf was placed in an identical control chamber at ambient humidity. Leaf chlorophyll content was monitored weekly with a SPAD meter for a maximum of 35 days or until leaves had chlorophyll contents below 25 μg cm^{-2}, the average level we associated with the onset of leaf senescence. In tropical and temperate trees, high humidity and ambient humidity control chambers were located in partially shaded branches to avoid overheating, and next to each other to reduce microenvironmental differences. Chlorophyll was monitored *in situ* with a SPAD meter every week for a maximum of 33 days or until leaves senesced or fell. Diurnal measurements of PAR (LI-250, LI-COR), relative humidity (SAM990DW digital sling psychrometer, McMaster-Carr), and ambient and leaf temperature (OS543, Omega) were performed on sunny days in both high humidity and control chambers. Quantum yield and electron transport rate light curves were determined with a Mini-Pam (Walz, Eichenring, Germany) to assess the effect of the high humidity treatment on photosynthetic light reactions.

Statistical analysis

Changes in chlorophyll content with leaf age were analysed with an ANOVA and *post hoc* Tukey tests (SPSS, IBM, Armonk, NY, USA). Differences in physiological and anatomical properties between young and old leaves, and the effect of high humidity on leaf chlorophyll with respect to control treatments, were examined with *t*-tests. Calibration curves were determined using the least-squares method.

Results

K_{leaf} *and chlorophyll content dynamics with leaf age*

Tomato leaf chlorophyll content remained relatively stable until leaves were an average of 18 days old, when values began to decline (Fig. 1). The onset of leaf senescence was defined as the point at which chlorophyll declined significantly below that of young leaves. Based on the linear regression of leaf age (days) and position (LNA), we estimated that leaf senescence was triggered at an average leaf age of 27 days (ANOVA, Tukey's *post hoc* test, $P < 0.05$; $n = 5$). K_{leaf} values tended to be highest in young leaves, where the average K_{leaf} was 20.9 ± 7.5 mmol H_2O m^{-2} s^{-1} MPa^{-1}. As leaves aged, K_{leaf} decreased to 64% of this value before the onset of senescence (ANOVA, Tukey's *post hoc* test, $P < 0.05$; $n = 5$). To assess the impact of aging on leaf hydraulics independently from leaf node with respect to the apex, we also compared leaves at a fixed node from the bottom of the plant but separated by a 25-day difference in age (Fig. S4). Similarly, K_{leaf} declined from an average of 19.4 ± 7.0 mmol H_2O m^{-2} s^{-1} MPa^{-1} to 9.1 ± 1.1 mmol H_2O m^{-2} s^{-1} MPa^{-1} (*t*-test, $P < 0.05$; $n = 5$).

The selected pre-dry season *A. excelsum* leaf cohort exhibited a significant reduction in chlorophyll content 95 days after completing leaf expansion (ANOVA, Tukey's *post hoc* test, $P < 0.05$; $n = 3$) (Fig. 1). This developmental age coincided with the median leaf longevity of 97 ± 75 days from the 1992–1994 censuses (unpubl. data by Wright *et al.*). The reduction in chlorophyll was significant when average values dropped to 85% of the maximum chlorophyll attained by the leaf cohort during the study period (ANOVA, Tukey's *post hoc* test, $P < 0.05$; $n = 3$). At full expansion, leaves had the highest average K_{leaf} levels during their lifespan (5.2 ± 0.6 mmol H_2O m^{-2} s^{-1} MPa^{-1}). As the leaf cohort aged, K_{leaf} declined steadily reaching less than 50% of the maximum before senescence was triggered (ANOVA, Tukey's *post hoc* test, $P < 0.05$; $n = 3$). Similarly, *P. trichantha* leaves senesced after 95 days of leaf expansion, also experiencing a reduction in K_{leaf} to 44% of a maximum 7.5 ± 1.0 mmol H_2O m^{-2} s^{-1} MPa^{-1} (ANOVA, Tukey's *post hoc* test, $P < 0.05$; $n = 3$). In contrast, temperate tree leaves in *A. saccharum* and *Q. rubra* did not show a reduction in hydraulic supply before senescence (Fig. 1). A significant decline in leaf chlorophyll marked the onset of senescence 91 and 107 days after expansion for *A. saccharum* and *Q. rubra*, respectively (ANOVA, Tukey's *post hoc* test, $P < 0.05$).

Tomato hydraulic conductance dynamics in leaf lamina and rachis with age

Leaf-specific hydraulic conductance of the rachis (K_{rachis}) dropped by 44% from young to old leaves (Fig. 2*a*), whereas leaflet hydraulic conductance ($K_{leaflet}$) declined by 36% (Fig. 2*b*) (*t*-test, $P < 0.01$ and $P < 0.05$ respectively; $n = 6$). K_{rachis} and $K_{leaflet}$ calculations involved measurements of total conductance (water flow per driving pressure, F ΔP^{-1}) and leaf area supplied by the vascular system. Each of these components can vary independently, so we assessed their individual impact on leaf hydraulics with leaf age. Although F ΔP^{-1} increased in the rachis with age (Fig. 2*c*) (*t*-test, $P < 0.01$; $n = 6$), in the lamina, it remained unchanged (Fig. 2*d*) (*t*-test, $P = 0.776$; $n = 6$). In contrast, leaf area supplied by the rachis xylem (Fig. 2*e*) and leaflet lamina area increased from young to old leaves (Fig. 2*f*) (*t*-test, $P < 0.01$ and $P < 0.05$ respectively; $n = 6$). Total vein length increased as leaves aged, but not at pace with leaf expansion resulting in a continuous decline in vein density for leaves of all ages from young to old leaves (Fig. 3).

Changes in petiole hydraulic properties with age were not associated with modifications in xylem vessel diameter distribution (Fig. S5). Most vessels were within the 20 μm range for both young and old leaves. Despite a slightly larger number of vessels in the 40 μm category in young leaves, the differences were not significant (*t*-test, $P = 0.097$ and $P = 0.438$, respectively; $n = 5$). Only one vessel in the 60 μm range was found in old leaves but none in young leaves. Furthermore, petiole percentage loss of conductance (PLC) due to xylem embolism

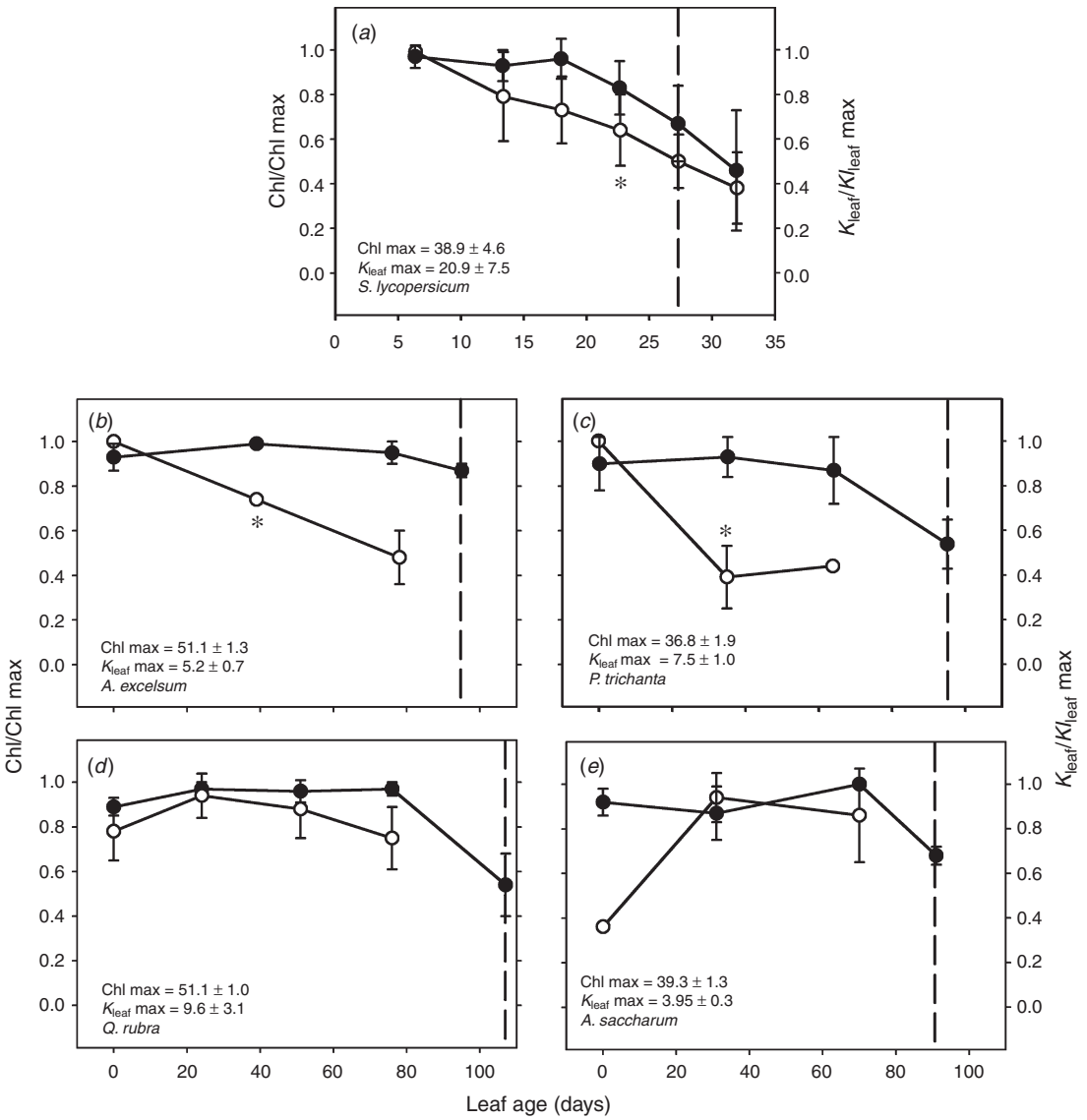

Fig. 1. Ratios of chlorophyll to maximum chlorophyll content (closed circles) and leaf hydraulic conductance (K_{leaf}) to maximum K_{leaf} (open circles) over the leaf's lifespan ($n = 3$–5) for (*a*) *Solanum lycopersicum*, (*b*) *Anacardium excelsum*, (*c*) *Pittoniotis trichantha*, (*d*) *Quercus rubra* and (*e*) *Acer saccharum*. Chlorophyll values were monitored until the onset of leaf senescence (dashed line), measured as a significant reduction in average chlorophyll content relative to the maximum level. Asterisk indicates a significant decline in K_{leaf}. Data are averages ± s.d. Maximum chlorophyll *a* + *b* content values are in expressed in μg cm^{-2}; K_{leaf} in mmol H_2O m^{-2} s^{-1} MPa^{-1}. Comparisons between leaf ages were tested with ANOVA, Tukey's *post hoc* test ($P < 0.05$).

was comparable for leaves of both ages (*t*-test, $P = 0.69$; $n = 6$), averaging 17 ± 14% and 22 ± 20% for young and old leaves, respectively. However, total petiole xylem area increased with leaf age from 0.54 ± 0.15 mm^2 to 1.23 ± 0.25 mm^2 (*t*-test, $P < 0.01$; $n = 5$).

Leaf hydraulic impact on gas exchange

A significant reduction in both *A* and g_s in tomato leaves (Fig. 4) was observed in leaves aged 22 days, after K_{leaf} dropped to 64% from its maximum, indicating a limitation of gas exchange by liquid phase transport. Stomatal conductance exhibited an average reduction of 59% before the onset of leaf senescence (ANOVA, Tukey's *post hoc* test, $P < 0.05$; $n = 5$). Despite reductions in K_{leaf} in the tropical trees *A. excelsum* and *P. trichantha* to less than half of the maximum K_{leaf}, there was no impact on gas exchange (Fig. 4). The slightly lower assimilation rates as leaves aged in *A. excelsum* were associated with changes in stomatal aperture. After leaf expansion, in the temperate tree *A. saccharum*, stomatal conductance was maintained almost unchanged; in *Q. rubra*, values initially increased before reaching a plateau (Fig. 4).

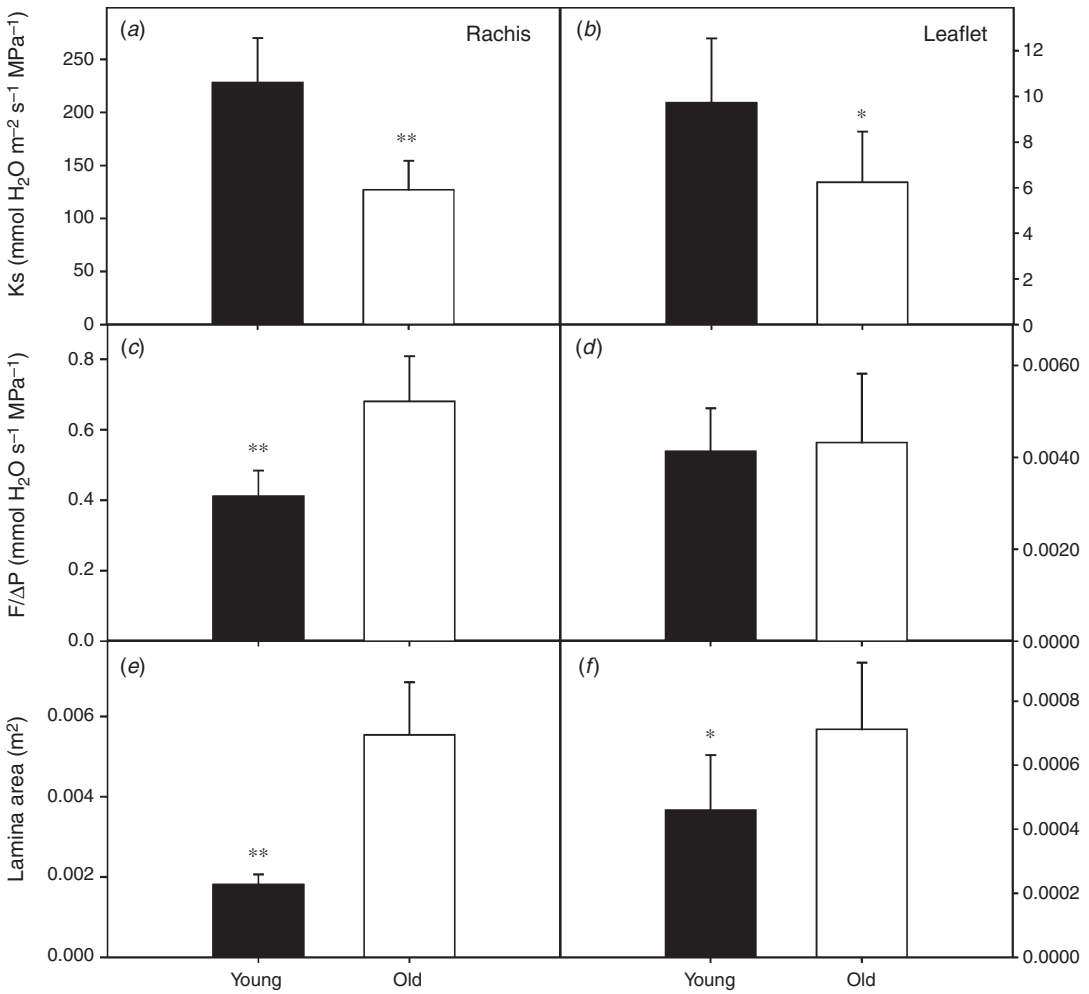

Fig. 2. Decline from young to old tomato leaves in (*a*) leaf-specific rachis hydraulic conductance (K_{rachis}) and (*b*) leaflet hydraulic conductance ($K_{leaflet}$) (*t*-test, $P < 0.01$ and $P < 0.05$ respectively). K_{rachis} is normalised by the leaf lamina area supplied by the rachis; $K_{leaflet}$ by leaflet area. Panels (*c*) and (*d*) represent changes from young to old tomato leaves in total conductance (flow of water per driving pressure, $F\ \Delta P^{-1}$) of (*c*) the rachis and (*d*) leaflets (*t*-test, $P < 0.01$ and $P = 0.776$ respectively). Panels (*e*) and (*f*) show the leaf lamina area supplied by (*e*) the rachis and (*f*) the leaflet area (*t*-test, $P < 0.01$ and $P < 0.05$ respectively). Bars are averages ± s.d. ($n = 6$).

Similarly, assimilation was relatively constant throughout the season with the exception of a slight increase in young leaves of *Q. rubra*.

Effect of transpiration downregulation on leaf senescence

Manipulation of leaf xylem flow rates via reducing transpiration in high humidity chambers induced earlier senescence in wild-type tomato leaves (*t*-test, $P < 0.05$; $n = 5$; Fig. 5). Leaves under high humidity had significantly lower chlorophyll levels at the end of the treatment, despite being younger than their control counterparts. Light reactions of photosynthesis were not affected by the humidity treatment, as indicated by quantum yield and electron transport rate light curves (Figs S6, S7). Reducing xylem transport delivery with canopy humidity chambers also impacted leaf chlorophyll dynamics with age in the tropical evergreen tree *A. excelsum* (*t*-test, $P < 0.05$; $n = 3$; Fig. 5). Leaves senesced and abscised after a week of applying the treatment during the dry season in February 2009 when trees were exchanging their leaves. In the dry season of the following year (January 2010), the individual studied responded with a decline in leaf chlorophyll but senescence occurred after 33 days of applying the humidity treatment. The later manipulation experiment was initiated when the tree was not flushing new leaves in a year characterised by a late leaf exchange of *A. excelsum* trees around the canopy crane (personal observation, data not shown). Quantum yield and electron transport rate light curves in high humidity and control treatments exhibited overlapping patterns (Figs S6, S7). The reduction in transpiration rates in the high humidity treatments did not impact the timing of leaf senescence in the deciduous tropical tree *P. trichantha* and the temperate trees *A. saccharum* and *Q. rubra* (Fig. 5). Despite leaves being exposed to mean diurnal VPD values in average five times lower than the ambient for more than a month (Fig. S8), chlorophyll content remained at similar levels in the canopy humidity and control chambers for these tree species. Quantum yield and electron transport rate

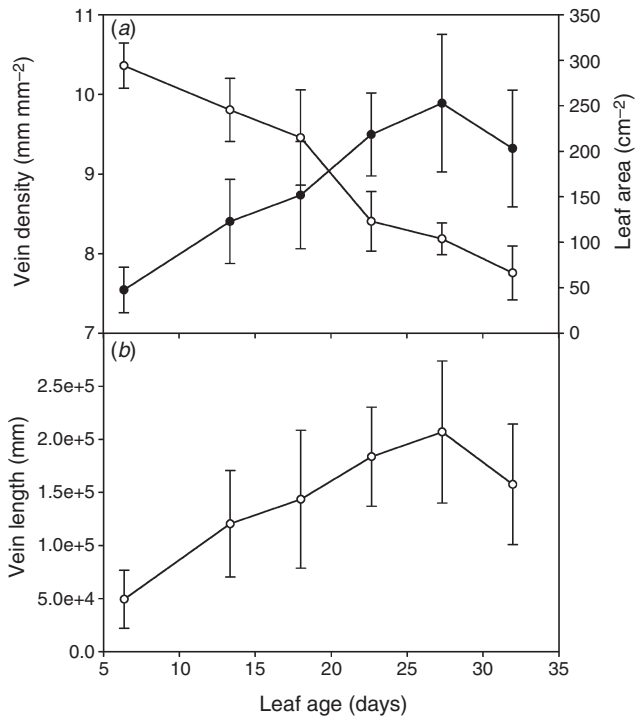

Fig. 3. Changes in average tomato (*a*) leaf vein density (white circles) and leaf area (dark circles) and (*b*) vein length from young leaves to the end of the leaf's lifespan ($n = 5–6$). Area values are normalised by the maximum leaf area within each individual. Error bars are s.d.

light curves were comparable in leaves of both treatments in *P. trichantha* and *A. saccharum* but not in *Q. rubra*, indicating a slight reduction of photosynthesis light reactions in canopy humidity chambers (Figs S6, S7). Diurnal patterns of leaf temperatures, air temperature and PAR were similar across treatments (Fig. S8). As expected, VPD values were lower in the humidity treatment.

Discussion

Age-related changes in leaf anatomy leads to K_{leaf} *downregulation before the onset of tomato leaf senescence*

Both K_{rachis} and $K_{leaflet}$ contributed to the decline in K_{leaf} before the onset of senescence in tomato leaves. K_{rachis} and $K_{leaflet}$ experienced an equivalent reduction in conductance of more than half of their initial capacity at early developmental stages. However, the hydraulic conductance of the rachis was more than 20 times higher than the lamina in both leaf age categories. Thus the conductance of the lamina may have constituted a leaf hydraulic bottleneck that influenced, to a larger extent, the age-dependent decline in K_{leaf}. The low hydraulic conductance of extraxylary pathways has been reported in angiosperm leaves (Cochard *et al*. 2004; Gascó *et al*. 2004). However, other studies have proposed a greater amount of conductance to water flow in the mesophyll relative to the xylem of leaf blades (Sack *et al*. 2002) and the minor contribution of rachis conductance to total fern frond water transport capacity (Lo Gullo *et al*. 2010), underscoring the need for a better understanding of hydraulic conductance partitioning within compound leaves.

In glasshouse-grown tomatoes, age-related changes due to endogenous factors rather than environmental stressors are the most parsimonious explanation of impaired leaf hydraulic function. Modifications in the hydraulic architecture of the lamina and rachis as leaves aged were associated with the observed decline in K_{leaf}. The maintenance of similar flow rates per driving pressure in the leaf lamina indicated that the total leaflet conductance across developmental stages remained unchanged. However, parallel reductions in leaf vein density with increases in leaf area with age suggest that the leaflet vascular system does not develop an adequate hydraulic supply to comply with lamina expansion. These changes in leaf anatomy with age echo findings that report that lower vein density in basal relative to apical leaves is associated with a decline in K_{leaf} (Nardini *et al*. 2008). Whether the relative hydraulic conductance of xylary to extraxylary pathways changes with leaf expansion in tomato remains unknown. Nardini *et al*. (2010) reported a concurrent trend of decrease in extravascular and vascular K_{leaf} with leaf area expansion accompanied by a shift from extravascular-dominated conductance at early stages of expansion to higher vascular conductance in fully expanded leaves. Ontogenetic changes in the hydraulic conductance of the leaf mesophyll have been associated with an increase in palisade and spongy cell density, which forces water to cross a higher frequency of cell membranes (Aasamaa *et al*. 2005), in which the majority of the resistance to leaf water flux may occur (Ye *et al*. 2008).

The reduction in K_{rachis} could not be attributed to changes of xylem flow rates per driving pressure, as the total rachis conductance increased with age, indicating a higher hydraulic supply. Neither petiole vessel size distributions nor PLC were associated with changes in K_{rachis} across leaf age categories, but PLC values reflected considerable amounts of embolism at both leaf developmental stages. Alternatively, vascular bundle radial hydraulic conductance, instead of conduit diameter, could have determined rachis hydraulics, as in ferns (Lo Gullo *et al*. 2010), but it remains to be tested if pit membrane permeability in angiosperm leaves is as great a contributor to leaf xylem resistance as it is in stems (Sperry *et al*. 2005). Our results contrast with those regarding *Populus tremula* Michx., in which midrib conduit size, but not cross-sectional area, was the driver in K_{leaf} changes during leaf ontogeny (Aasamaa *et al*. 2005). Only larger xylem conductive area in old tomato leaves could explain the increase in total rachis conductance. However, increased xylem hydraulic supply in the rachis in old leaves was not sufficient to compensate for leaf area expansion, leading to a net K_{rachis} decline.

Leaf vascular supply influences the onset of age-dependent leaf senescence in tomato

Many studies have recognised that leaf senescence is an age-dependent process (Lim *et al*. 2007). We propose that in tomato, K_{leaf} can act as an age-dependent signal determining the timing of leaf senescence by limiting gas exchange and thus xylem-transported compound flow into leaves. The early induction of leaf senescence by transpiration downregulation in canopy humidity chambers, independent of light levels, suggests a role

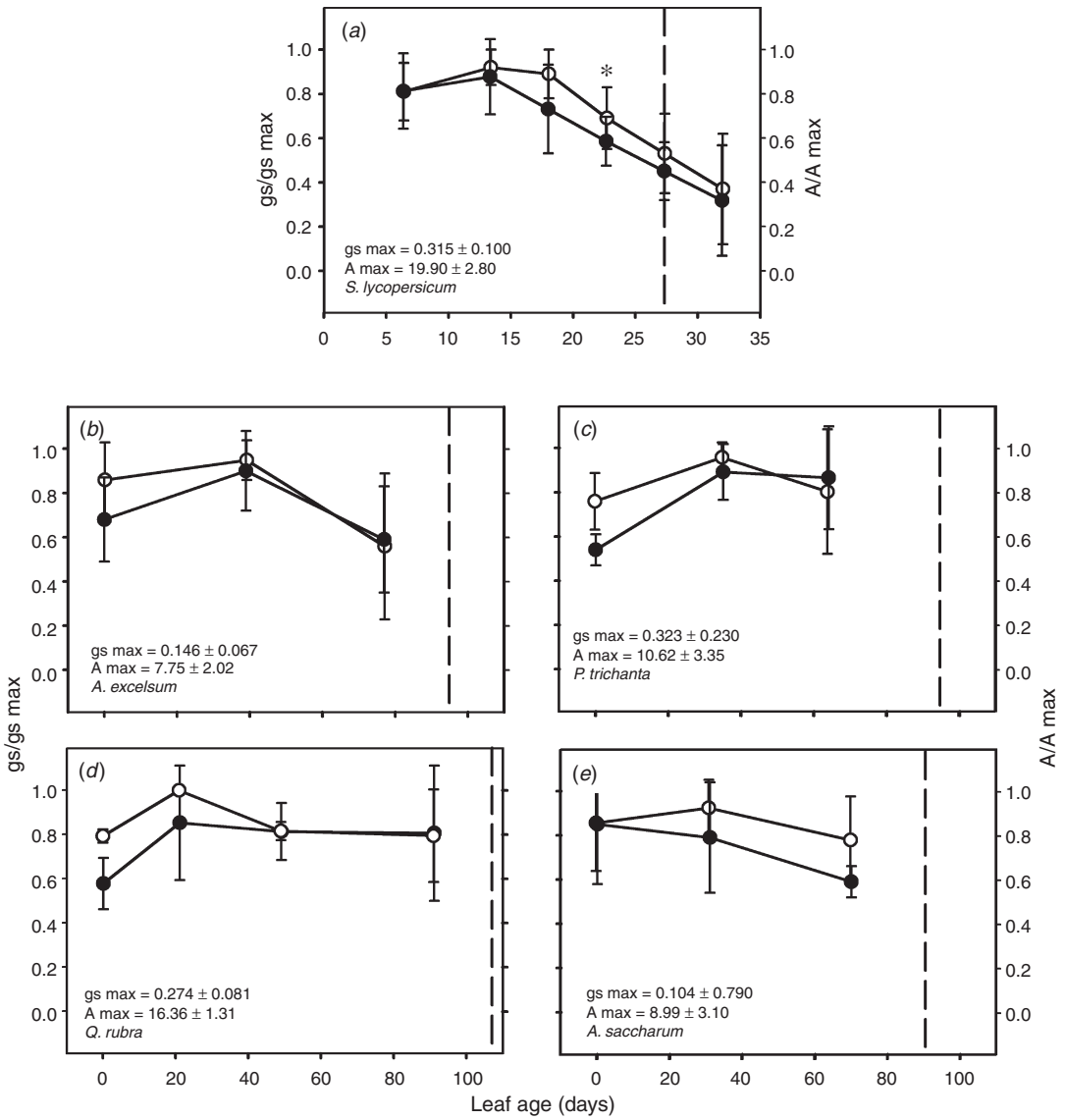

Fig. 4. Patterns of stomatal conductance (g_s, closed circles) and light saturated assimilation (A, open circles) with leaf age for (*a*) tomato, (*b*) *Anacardium excelsum*, (*c*) *Pittoniotis trichantha*, (*d*) *Quercus rubra* and (*e*) *Acer saccharum*. Maximum values are in expressed in mol H_2O m^{-2} s^{-1} for g_s and in mmol CO_2 m^{-2} s^{-1} for *A*. Data are averages of leaf gas exchange values with the respective s.d. ($n = 3$–5).

of xylem-transported compounds in determining leaf senescence in tomato. The age-dependent decline in K_{leaf} of tomato plants associated with a reduction in gas exchange also indicates a limitation to xylem compound delivery. Consistent with this view, previous studies have shown that the delivery of xylem-transported compounds such as cytokinins are dependent on transpiration rates in tobacco and *A. thaliana* (Boonman *et al.* 2007; Boonman *et al.* 2009). Similarly, rootstock-mediated manipulations of xylem-transported cytokinins in tomato plants point to their role in delaying leaf senescence under high salinity conditions (Ghanem *et al.* 2008; Albacete *et al.* 2009).

K_{leaf} dowregulation with age was driven by a reduction in leaf vein density associated with leaf lamina expansion. As leaves age, neither the xylary pathways nor the lamina vascular system develop the hydraulic supply required by a larger leaf conductive area. Hydraulic limitations in the leaf lamina have been shown to play a key role in determining leaf size and shape (Zwieniecki *et al.* 2004; Carins Murphy *et al.* 2012; Zhang *et al.* 2012). Similarly, as K_{leaf} began to limit gas exchange in tomato leaves, lamina expansion ceased. Constraining K_{leaf} dynamics to leaf expansion provides a simple but consistent age-dependent mechanism to control the timing of leaf senescence via regulation of the flow of xylem-transported compounds. An adaptive advantage of the proposed mechanism is that it may allow plants to recognise the developmental age of leaves for nutrient translocation during new leaf growth or plant reproduction.

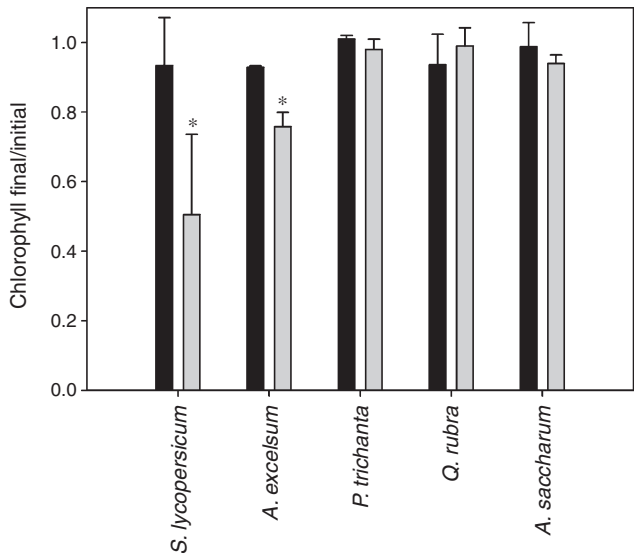

Fig. 5. Reduced transpiration impacts chlorophyll content in *Solanum lycopersicum* and the tropical evergreen tree *Anacardium excelsum* (*t*-test, $P < 0.05$) but not in the tropical deciduous *Pittoniotis trichantha*, or the temperate deciduous *Acer saccharum* and *Quercus rubra*. Data represent the average final over initial ratio of leaf chlorophyll ± s.d. ($n = 3$–6) for leaves in high humidity (grey bars) and control chambers (black bars). Final values were taken before leaf fall or up to a maximum of 35 days after initiating the treatment.

K_{leaf} does not affect leaf senescence in tropical A. excelsum and P. trichantha and temperate A. saccharum and Q. rubra trees

Leaf vascular influence on senescence appeared consistent with K_{leaf} reduction before a decline in chlorophyll content in *A. excelsum* and *A. trichanta*. However, there was no gas exchange downregulation with leaf age, indicating that K_{leaf} does not limit the flow of xylem-transported compounds. Tropical tree species vary in the degree of gas exchange response to K_{leaf} decline. Some show a strong correlation between K_{leaf} and their gas exchange recovery during rehydration; in others, stomatal conductance is insensitive to artificially depressed K_{leaf} (Brodribb and Holbrook 2007; Blackman *et al*. 2009; Brodribb and Cochard 2009). The insensitive species have been linked to a linear decline in K_{leaf} with leaf water potential, whereas more sensitive species exhibit a sigmoidal curve (Brodribb and Holbrook 2007). *A. excelsum*'s and *P. trichantha*'s K_{leaf} average values below 7.5 mmol H_2O m^{-2} s^{-1} MPa^{-1} fall within the low range reported for tropical woody angiosperm species (Brodribb *et al*. 2005; Sack and Holbrook 2006), where there should be a strong correlation between K_{leaf} and maximum carbon assimilation (Brodribb *et al*. 2005). However, we recorded only a slight or no decrease in assimilation and stomatal conductance.

In the tropical deciduous tree *P. trichantha*, reducing leaf transpiration in high humidity chambers had no impact on chlorophyll dynamics; however, in evergreen *A. excelsum* trees, early senescence was triggered only during leaf exchange events in the dry season. Reducing leaf xylem transport delivery is, therefore, not a sufficient condition for the induction of senescence. Future studies should examine the hypothesis of a nitrogen sink requirement for the occurrence of age-dependent leaf senescence in *A. excelsum*. It is well known that the leaves of herbaceous plants grown at high nitrogen tend to senesce later (Ono *et al*. 2001). Delaying leaf senescence using nitrogen fertilisation has been reported in the temperate tree *Populus trichocarpa* Tor. & Gray (Sigurdsson 2001). However, how xylem-transported nitrogen affects tropical leaf phenology is not well understood. According to this framework, the leaf senescence program could be activated in evergreen tropical trees when sink organs like young leaves, flowers or fruits initiate their development. Evidence supporting this hypothesis comes from the seasonal leaf dynamics and reproductive phenology censuses from 1992 to 1994, recording leaf mortality increases together with the induction of new leaf and reproductive growth in *A. excelsum* (unpubl. data by Wright *et al.*). The physiological mechanisms regulating leaf senescence in tropical trees with diverse phenological strategies remain to be explored (Giraldo and Holbrook 2011).

Our results indicate that the mechanisms regulating leaf senescence of the temperate tree species *A. saccharum* and *Q. rubra* do not involve changes in leaf vascular supply. Average K_{leaf} values were constant before senescence and were comparable to those reported with the vacuum technique (Lo Gullo *et al*. 2005) but lower than measured with evaporative flux method (Sack *et al*. 2002). The divergences could be a consequence of this study's working range of more negative water potentials and higher flow rates. Our findings also contrast with leaf hydraulic dynamics studies of *Q. rubra* trees planted in the Mediterranean region in which a 70% drop in seasonal K_{leaf} occurred before any visible changes in leaf colouring (Lo Gullo *et al*. 2005), suggesting a plastic response of leaf hydraulic properties to seasonal variations in environmental conditions. Furthermore, reduction of leaf transpiration rates in canopy humidity chambers lasting more than a month did not trigger early leaf senescence. Together, our results indicate that xylem delivery does not impact the timing of leaf senescence in these temperate tree species.

Nevertheless, we cannot rule out the proposed hydraulic mechanism for regulating leaf senescence via xylem compound delivery for other temperate tree species. Salleo *et al*. (2002) identified a reduction in K_{leaf} during the photosynthetic season preceding any visible changes in *Castanea sativa* L. leaf colouring, leading the authors to hypothesise that widespread xylem blockage in both the stem and leaves due to the formation of embolism, followed by tyloses growing into the conduits, could initiate leaf shedding in this temperate tree species. It has also been proposed that ring-porous temperate hardwood species that lose early wood vessels every year (Zimmermann 1983) tend to senesce earlier in the autumn than their diffuse-porous counterparts due to summer drought-induced loss in xylem function (Wang *et al*. 1992). Seasonal reductions in stem and leaf hydraulic supply have been linked to strong coordination with stomatal conductance (Lo Gullo *et al*. 2005) but the impact of variations in temperate trees' hydraulic resistance on the delivery of xylem-transported compounds via gas exchange limitation remains poorly understood.

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