grown axenically *in vitro* using supplemented RPMI 1640 medium, and at intervals after the stationary phase (21 days) they were passaged into new cultures containing each drug or both drugs (hygromycin, 150 µg ml⁻¹; G418, 120 µg ml⁻¹). Second, mammalian cell (Vero) monolayers were infected with P1-*hyg* and P2-*neo* using stationary-phase, mixed axenic cultures containing epimastigotes and metacyclic trypomastigotes. Trypomastigotes from pseudocysts were recovered periodically between days 7 and 28, and grown as epimastigotes in axenic culture for drug sensitivity tests. Third, triatomine bugs were membrane fed on mouse blood containing P1-*hyg* and P2-*neo* trypomastigotes derived from Vero cell monolayers. Bugs were dissected 25–30 days later, *T. cruzi* was re-isolated by culture on biphasic blood agar, and was passaged into axenic culture to obtain sufficient organisms for testing drug sensitivities. Last, groups of three immunocompromised (SCID) mice were inoculated with a mixture of faeces from triatomine bugs carrying P1-*hyg* or P2-*neo*. Populations were subsequently retrieved from infected mice into axenic culture and placed under drug pressure.

Determination of phenotype and genotype

DNA purification was carried out by phenol/chloroform extraction and ethanol precipitation or using DNeasy (Qiagen). Amplification reactions used the following conditions: denaturation for 5 min at 94 °C, then 30 cycles of 94 °C (1 min), 50–62 °C (1 min; depending on the primer T_m) and 72 °C (1 min per 1,000 bp), followed by 10 min at 72 °C. Primers are described in Supplementary appendix 6. PGM phenotype determination and RAPD analysis were as described previously[6]. Episomes were detected by multiplex amplification using primers designed to the hygromycin phosphotransferase and neomycin phosphotransferase genes. For karyotype analysis we used a Bio-Rad CHEF Mapper with an autoalgorithm for separation of 0.4–2.2-Mb fragments, followed by Southern blotting and hybridization with radiolabelled probes.

The following DNA sequences were amplified, some with *Taq* Extender; Stratagene: (1) *tpn*1[19] (tryparedoxin: GenBank accession number AF106855; 435 bp); (2) *gpi* (1,038 bp); (3) putative *pgm* (380 bp) (TIGR database (http://www.tigr.org) accession number TC1375), identified by similarity between human and putative *Leishmania pgm*; (4) *tcp*, an intergenic region (760 bp, including gaps) flanked by 3′ *tcp17* and partial 5′ *tcpgp2* (ref. 20); (5) a mitochondrial locus (1,078 bp) spanning the maxicircle-encoded genes cytochrome oxidase subunit II (*coII*) and *nd1* (ref. 3); and (6) *dhfr-ts* (1,042 bp)[3]. PCR products from all loci were cloned into pGEM T (Promega), except where no heterozygous alleles were detectable (mitochondrial DNA[3] of progeny; *gpi* of some isolates). For each isolate a minimum of three (*gpi*) and either three or six (*tcp*) clones were sequenced on a capillary sequencer (Beckman) or an ABI 377 using relevant kits.

Genotypes were also determined at eight microsatellite loci[13], with primers labelled with FAM, NED and HEX, and sized against the ROX 500 marker (ABI) using an ABI 377. An additional 12 microsatellite loci were identified by searching the *T. cruzi* genome database for dinucleotide repeats (TIGR database; see also Supplementary appendix 7). Genescan and Genotyper software (Applied Biosystems) were used to automate measurements of allele length. All microsatellite loci were amplified from P1-*hyg* and P2-*neo* and their double-drug-resistant progeny using standard conditions; a subset (MCLE01, MCLEF10, MCLEG10, SCLE10 and SCLE11 (ref. 13), and A427, A831.3, E801, J356 and N060 (Supplementary appendix 7)) was amplified from all reference strains and field isolates (Supplementary appendix 8).

Phylogenetic analysis

Nucleotide sequences were aligned using Clustal X[27] then edited by hand, and are available on request. Primarily, sites of recombination were examined by bootscan analysis[28] using the Kimura two-parameter model. Recombination sites were investigated further by maximum likelihood breakpoint analysis[29]. All maximum likelihood parameter estimates comprising a four-category gamma distribution and a transition/transversion ratio (κ) were obtained with the tree bisection reconnection (TBR) heuristic search (where reconnection limit = 4) using PAUP* 4.0b (D. L. Swofford). Each data set of field isolates and reference strains was also subject to refined split decomposition analysis (Kimura three-parameter model)[30].

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Water transport in plants obeys Murray's law

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The optimal water transport system in plants should maximize hydraulic conductance (which is proportional to photosynthesis[1–5]) for a given investment in transport tissue. To investigate how this optimum may be achieved, we have performed computer simulations of the hydraulic conductance of a branched transport system. Here we show that the optimum network is not achieved by the commonly assumed pipe model of plant form[6–8], or its antecedent, da Vinci's rule[9,10]. In these representations, the number and area of xylem conduits is constant at

every branch rank. Instead, the optimum network has a minimum number of wide conduits at the base that feed an increasing number of narrower conduits distally. This follows from the application of Murray's law, which predicts the optimal taper of blood vessels in the cardiovascular system[11]. Our measurements of plant xylem indicate that these conduits conform to the Murray's law optimum as long as they do not function additionally as supports for the plant body.

The idea that organisms may be optimally designed has fuelled considerable research and controversy[12–15]. Both animals and plants require extensive and expensive transport systems, for which an optimal design presumably provides a selective advantage. In plants, transport through xylem has allowed growth in height and colonization of diverse habitats[16], and must be extensive because photosynthesis entails an exchange of large quantities of internal water for atmospheric carbon dioxide. Plants use capillary suction to transport water from the roots to the leaves without using metabolic energy, and the xylem conduits themselves are cheap to maintain, being dead at maturity. However, the numerous thick-walled conduits require considerable carbon investment. This cost, combined with the benefit to photosynthesis of optimizing xylem design, implies that it would be advantageous for plants to maximize hydraulic conductance per vascular investment, and hence, to obey Murray's law[11].

In 1926, Murray[11] developed a theory of optimal cardiovascular design that solves for the sizes of blood vessels from the aorta through progressive branch points to the capillaries that maximize the hydraulic conductance of flow through the vascular network for a fixed investment in blood and vessel volume. Under ideal conditions, the optimal design equalizes the sum of all radii cubed (Σr^3) at all points along the flow path if the volume flow rate (Q) of the blood is conserved within the vascular system and the flow is laminar[11]. This result, known as Murray's law, fits data better than equalizing the sum of radii raised to the second or fourth powers[17–19]. Surprisingly, this law has not been systematically applied and tested in plant vasculature[20].

Murray's law is applicable to plant xylem given the following four conditions. (1) The steady-state xylem Q is constant along the flow path. With the exception of the absorbing roots and the minor leaf veins, there will be no net loss of water from the xylem to surrounding tissues under steady-state conditions. (2) Xylem hydraulic conductance is proportional to conduit radius raised to the fourth power. This is the Hagen–Poiseuille prediction for laminar flow through cylindrical capillaries, which are appropriate

approximations for low-velocity xylem flow. (3) Conduit wall volume is proportional to total conduit volume. Murray assumed that the major volume cost was in the blood, and that the thin walls of blood vessels were negligible. In plants it is the opposite: the xylem water is cheap, but the conduit walls are costly. Murray's law is still valid, as long as the conduit wall volume scales directly with total conduit volume. The wall volume/conduit volume is proportional to the cavitation resistance of xylem[21], as is necessary to withstand the compressive forces on the wall caused by negative pressure. Thus, for a given cavitation resistance throughout the xylem network, Murray's law should apply. (4) The xylem conduits must function primarily in transport, as opposed to providing an additional mechanical support role. When conduits are serving both functions (as in conifer shoots or diffuse-porous trees), the optimization criteria must include mechanical as well as hydraulic considerations, and Murray's law is inappropriate.

Murray's law by itself is not sufficient to characterize the conductance versus investment trade-off in xylem, because of fundamental structural differences between the vasculature of animals and plants. Figure 1a illustrates the single-branched tube of the animal network. As the single parent tube at rank 0 (for example, the
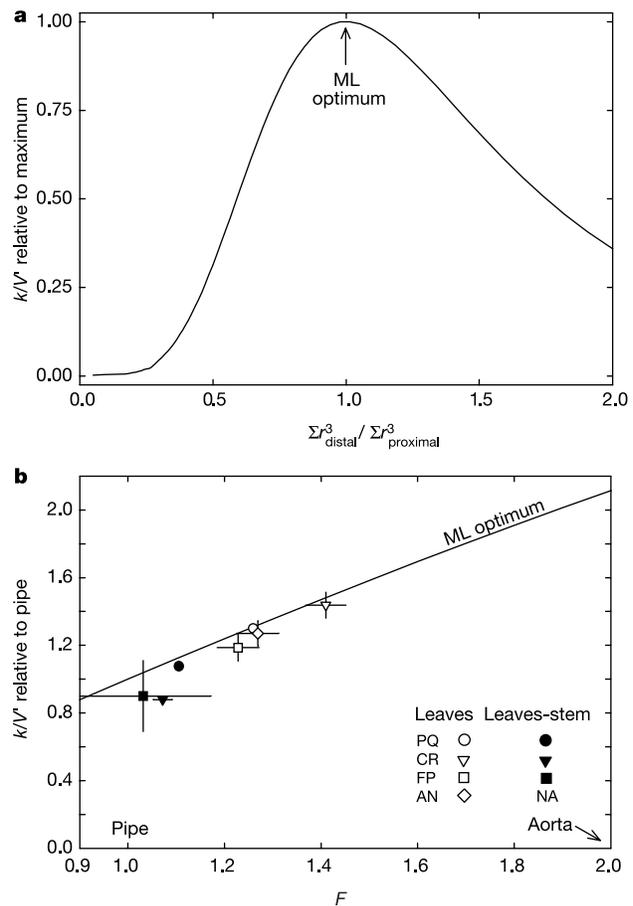
Figure 2 Murray's law. **a**, The Murray's law (ML) optimum. The line shows relative hydraulic conductance (k) per fixed volume (V') plotted against the ratio of the sum of conduit radii cubed (Σr^3) of distal:proximal ranks in a modelled vascular network. The maximum k/V' occurs at a Σr^3 ratio of 1 according to Murray's law regardless of the conduit furcation number (F). **b**, The Murray law optimum versus F. The optimum is set to 1 for $F = 1$ (pipe model). The greater efficiency of wide, proximal conduits in systems with higher F increases the hydraulic conductance for a given volume over the pipe model. The symbols show the measured F relative to the mean Murray's law optimum within leaves (open symbols, ±s.d.) and comparing petiolules with stems (filled symbols, ±s.d.). PQ, *Parthenocissus quinquefolia* (vine); CR, *Campsis radicans* (vine); FP, *Fraxinus pensylvanica* (ring-porous tree); and AN, *Acer negundo* (diffuse-porous tree).

Figure 1 Transport networks. **a**, Vascular networks in animals; **b**, **c**, plant xylem. The most proximal rank (rank 0) branches to form progressively distal ranks (for example, rank 1). In the animal cardiovascular system (**a**), a single continuous tube ramifies and the number of branches increases by an integer factor (the conduit furcation number, F) between ranks. In the plant system, tubes are not continuous, but divided into conduits. Hundreds of conduits run in parallel within each branch (only a few are shown for clarity). In the 'pipe model' (**b**), the number of conduits is identical regardless of rank, and $F = 1$. In the 'aorta model' (**c**) the number of conduits increases with the number of branches by an integer factor as in the animal system, and $F = 2$. The pipe and aorta models are points in a continuum, because with hundreds of conduits in a single branch, F can be a fraction.

Figure 3 ANOVA P values comparing the sum of the conduit radii raised to the x power (Σr^x) between petiolule versus petiole ranks of compound leaves. The P values are shown versus the exponent x. As the P value approaches 1, differences in Σr^x between ranks are minimized. Although Murray's law predicts the maximum P value at $x = 3$, the law cannot be rejected if P at $x = 3$ is above 0.05 (dashed line). None of the P values at $x = 3$ were below 0.05.

aorta) branches into daughter tubes at rank 1, the number of tubes increases by a predictable integer factor, here referred to as the conduit furcation number (F). Such a system would be disastrous in plants because the xylem sap is under negative pressure, and a single injury would fill the entire network with air. Instead, the main stem of a plant at rank 0 has numerous conduits arranged in parallel and series, which can confine any dysfunction to a single conduit. Unlike the animal system, there is no constraint on F. For example, in Fig. 1b, the number of conduits stays the same across ranks; this configuration has become known as the "pipe model"[6,22] ($F = 1$). Alternatively, the conduits can increase with branch rank by the same integer factor as in the animal system (Fig. 1c, 'aorta model'), but with multiple 'aortas' at rank 0 ($F = 2$). The pipe versus aorta distinctions are points in a continuum because F need not be an integer.

How does the pipe versus aorta configuration influence the trade-off between vascular conductance and investment? Regardless of F, the modelled maximum hydraulic conductance per vascular volume always occurs when Σr^3 is conserved throughout the system (Fig. 2a). This can also be shown by incorporating F in the derivation of Murray's law (Supplementary Information). However, the absolute value of the optimum increases with F (Fig. 2b). The pipe model yields substantially lower hydraulic conductance than the aorta model. The aorta model achieves greater conductance per volume by exploiting the increased efficiency of fewer, wider conduits in the proximal ranks.

The data in Fig. 2 raise the question of where real plants fall with respect to optimal conductance per volume. We put forward the hypothesis that xylem networks that meet the four assumptions of Murray's law should fall on the optimum with an $F > 1$ to exploit

the efficiency of fewer, wider conduits proximally. This hypothesis was tested in three contexts where the xylem conduit network does not perform a major mechanical support function: compound leaves, vines, and ring-porous trees (Table 1). Leaves are supported by turgor pressure and collenchyma, not xylem conduits. Compound leaves were chosen because there is no change in Q between petiolule and petiole ranks, unlike between ranks within leaf blade venation where Q is lost to transpiration. Vines are structural parasites, being supported by other plants or structures. In ring-porous trees, fibres provide the bulk of the structural support and the vessels comprise a relatively small fraction of the wood[21].

The data for compound leaves were consistent with Murray's law. Figure 3 shows the results of the petiolule versus petiole comparison in four species (Table 1). The Σr^x comparisons across ranks yielded maximum values of P for x between 2.4 and 3.2. In no species was the P value for $x = 3$ less than 0.05 (Fig. 3, dashed line), meaning that the Σr^3 was not significantly different between ranks. The shoot xylem of vine and ring-porous tree species was also generally consistent with Murray's law. Two out of the three petiolule versus stem comparisons had a P value for $x = 3$ greater than 0.05. The one deviant was only slightly different with the closest x at $P > 0.05$ equal to 2.85 (data not shown).

The proximity of each comparison to the Murray optimum is shown by the symbols in Fig. 2b. All comparisons had $F > 1$ as predicted, and there was a significant trend for xylem closest to Murray's law to have the greatest F value (Kendall's coefficient of rank correlation, $P < 0.05$ (ref. 23), symbols in Fig. 2b). The greatest F of 1.45 fell short of the aorta model ($F = 2$), perhaps because the increase in efficiency is offset by the reduction of conduit number in proximal stems and their increasing diameter (Table 1)—both factors can render the system vulnerable to failure[24]. Importantly, the situations in which F was greatest always occurred in the leaves, which are constructed specifically as disposable organs. Leaf versus stem comparisons tended to have greater Σr^3 in proximal versus distal ranks, and lower F (Fig. 2b, filled symbols).

Despite considerable interest in the adaptive significance of plant form, we are still far from understanding it. Quantifying the applicability of Murray's law is just one piece of the puzzle. Nevertheless, it improves upon Leonardo da Vinci's 'rule' that the total cross-sectional area of a tree's stems must be constant across branch ranks (area-preserving branching) to adequately supply the leaves[9,10]. The Murray optimum at $F > 1$ requires an increase in conduit cross-sectional area along the branched flow path (Table 1). The interaction between hydraulic supply and mechanical support of plant canopies undoubtedly underlies the distribution of supply versus support tissue in shoot structure. The two requirements are in conflict, because the optimal distribution of a fixed volume of tissue to support the tallest column requires a reduction in tissue area with height[25,26].

The allometry of plant form predicts large-scale biological consequences—from the scaling between physiological processes and body size to the energy budgets of ecosystems. Recent work shows how a very specific plant vascular organization (the pipe model with $F = 1$) and branching pattern (exact self-similarity with no apical dominance) that functions at the equivalent of the Murray's law

Table 1 Conduit number, mean radii and area

Rank	PQ (vine, $n = 3$)			CR (vine, $n = 3$)			FP (ring-porous, $n = 3$)			AN (diffuse-porous, $n = 4$)		
	CN	r (µm)	CA	CN	r (µm)	CA	CN	r (µm)	CA	CN	r (µm)	CA
Petiolule	10.4 (0.4)	17.08 (0.3)	2.6 (0.1)	17.7 (3.0)	13 (1.0)	2.5 (0.7)	3.0 (1.2)	19.9 (1.2)	1.1 (0.3)	1.8 (0.1)	18.4 (0.9)	1.2 (0.1)
Petiole	5.2 (0.1)	21.7 (0.6)	2.1 (0.2)	3.5 (0.6)	21.7 (1.8)	1.4 (0.3)	1.5 (0.6)	26.7 (2.1)	1 (0.3)	1 (0)	23.8 (2.3)	1 (0)
1-yr bud scar	1 (0)	35.8 (1.9)	1 (0)	1 (0)	32.6 (5.6)	1 (0)	1 (0)	24 (5)	1 (0)	NA	NA	NA

Branch ranks, relative change in total conduit number versus most proximal rank (CN), average conduit radius (r), and relative change in total conduit area versus most proximal rank (CA) for each species. The standard deviation from the mean of the individuals is listed in parentheses. PQ, *Parthenocissus quinquefolia* (vine); CR, *Campsis radicans* (vine); FP, *Fraxinus pensylvanica* (ring-porous tree); AN, *Acer negundo* (diffuse-porous tree). n, number of individuals measured per species. NA, not applicable. The 1-yr-old wood of *A. negundo* was not measured because the wood area is largely composed of conduits, which should contribute to the structural support of the plant.

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optimum can be extrapolated to explain metabolic scaling rules in biology[6–8]. The challenge is to relax the unrealistic structural assumptions of these models and evaluate hydraulic and mechanical optima. We have seen that F is not necessarily 1, and we have determined the applicability of Murray's law to xylem. A challenge for the future is to develop a better understanding of the interaction between tree biomechanics, hydraulics and growth. ☐

Methods

A computer programme calculated the hydraulic conductance per fixed vascular volume of a branched transport network. We modelled a constant-volume vascular network with specified number of ranks, a branch furcation number, B ($B = 2$, dichotomous, $B = 3$, trichotomous, and so on), and F. We standardized F to equal 1 for the pipe model and 2 for the aorta model regardless of B ($F = [(F' - 1)/(B - 1)] + 1$, where F' is the raw conduit furcation number). The network had constant F across ranks, and constant fractional taper in conduit diameter across ranks (diameter was constant within rank). The number of conduits at the distal rank was one per branch—the minimum required to vascularize the system. Rank length was unity, because the Murray's law solution is independent of branch length. To generate Fig. 2a, we varied conduit taper and computed the network conductance. To generate Fig. 2b, conduit taper was set to match Murray's law, and network hydraulic conductance computed as F was changed.

Conduit measurements were made on species with compound leaves (Table 1) collected in the greater Salt Lake City (Utah) area (40° 46′ N, 111° 58′ W). Three to four individuals per species were collected from the same habitat, and were similar in size. Upon collection, plants were perfused with 0.05% basic fuchsin to determine which xylem conduits were functional, and only these were measured. Between 2,000 and 6,000 conduits were measured per individual. Conduit radii were measured at the petiolule and petiole rank for leaves, and at the current year's annual bud scar in stems (Table 1).

Conduit statistics per rank had to be estimated, given the large numbers of conduits involved. In leaves, all conduits in petioles and petiolules were measured in a sample of ≥5 leaves. Each measured leaf provided a Σr^x per leaf area which was multiplied by total leaf area to estimate rank Σr^x. In secondary stem xylem, conduits in ≥3 radial sectors per branch were measured, with each sector yielding a Σr^x per sector area. Each subsample was multiplied by total xylem area of the rank to obtain a rank Σr^x estimate. These multiple Σr^x estimates per rank were incorporated into the analysis of variance (ANOVA).

To test Murray's law, the Σr^x of the distal-most rank (petiolule) was compared against the Σr^x of each proximal rank (petiole, stem xylem at first year bud scar). To test the conservation of Σr^3, x in each of the rank estimates of Σr^x was incremented from 1 to 5. Raising the Σr^x to relatively high x resulted in frequency distributions that were not normally distributed, so the data were log-transformed for statistical analyses. For each species we used a 2-way ANOVA with the Σr^x estimates as the dependent variable, the distal-most versus proximal rank pair as the fixed factor, and the replicate individuals as a random factor (for the petiolule: petiole comparison there were 26 degrees of freedom for PQ, CR and FP; and 45 for AN (see Table 1 for nomenclature); for the petiolule:1-yr-old wood comparison, there were 25 degrees of freedom for PQ, 21 for CR and 43 for FP[27] (SPSS Version 8.0.0 (SPSS, Chicago, 1998)). Increased similarity in estimates of Σr^x between ranks resulted in P-values approaching 1. For each paired comparison we used a 2-way ANOVA to identify the range of x over which Σr^x between ranks was not significantly different at $P \geq 0.05$. If this range of x did not include 3, Murray's law was rejected for that comparison.

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Extra-embryonic function of Rb is essential for embryonic development and viability

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The retinoblastoma (*Rb*) gene was the first tumour suppressor identified[1]. Inactivation of *Rb* in mice results in unscheduled cell proliferation, apoptosis and widespread developmental defects, leading to embryonic death by day 14.5 (refs 2–4). However, the actual cause of the embryonic lethality has not been fully investigated. Here we show that loss of Rb leads to excessive proliferation of trophoblast cells and a severe disruption of the normal labyrinth architecture in the placenta. This is accompanied by a decrease in vascularization and a reduction in placental transport function. We used two complementary techniques—tetraploid aggregation and conditional knockout strategies—to demonstrate that *Rb*-deficient embryos supplied