spring equinox (Fig. 2a; 0° and 2° S), when the advances in sunset time are slightly smaller (7 and 7.6 min versus 5.9 and 5.4 min). Implicitly, cumulative changes in sunset time of 5–7 min over 20 days are sufficient to induce flowering at the Equator.

In view of the small maximum changes in sunset time near the Equator (5–7 min over 20 days) it seems likely that signals sufficiently large to trigger flowering or vegetative bud break occur only during periods of maximum change (Fig. 1 d, f), that is, around the equinoxes (Fig. 2, grey bars) or solstices (Fig. 2d). If so, the observed staggered flowering times (Fig. 2a) could be primarily attributable to differences in the duration of flower development induced by perception of the photoperiodic signal. Flowering during an inductive period might indicate rapid flower emergence from resting buds, as in several *Miconia* species (Fig. 2a; 2.5–3.8° S). In other species, trees may flower months after the inductive period because the flowering signal causes the vegetative shoot apex to change into a large, branched inflorescence supporting many flowers[4] (Fig. 2a; 4° N *Montanoa*). It remains to be explained why some species flower only during or shortly before one of the two induction periods and why many trees do not flower every year.

This is the first study that both confirms synchronous flowering in rainforest tree species near the Equator and proposes a timing mechanism. Synchronous flowering at the same time each year has long been noticed in Amazonian trees such as *Miconia* (Fig. 2a; 2.5° S), but studies of the phenomenon focused on the evolutionary consequences of staggered synchronous fruiting, for example, for frugivorous birds[14]. Photoperiodic control of vegetative development and flowering in tropical trees evolved in response to different adaptive pressures[3,5,6]. In tropical rainforests with an equitable climate, it may have evolved in response to the need to synchronize flowering to achieve cross-pollination in spite of low population densities. □

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Gene transfer to plants by diverse species of bacteria

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***Agrobacterium* is widely considered to be the only bacterial genus capable of transferring genes to plants. When suitably modified, *Agrobacterium* has become the most effective vector for gene transfer in plant biotechnology[1]. However, the complexity of the patent landscape[2] has created both real and perceived obstacles to the effective use of this technology for agricultural improvements by many public and private organizations worldwide. Here we show that several species of bacteria outside the *Agrobacterium* genus can be modified to mediate gene transfer to a number of diverse plants. These plant-associated symbiotic bacteria were made competent for gene transfer by acquisition of both a disarmed Ti plasmid and a suitable binary vector. This alternative to *Agrobacterium*-mediated technology for crop improvement, in addition to affording a versatile 'open source' platform for plant biotechnology, may lead to new uses of natural bacteria–plant interactions to achieve plant transformation.**

Agrobacterium tumefaciens is a ubiquitous soil bacterium that induces galls on plants. The discovery that this gall formation is due to integration into the plant genome of bacterial DNA (T-DNA) laid the foundations for plant biotechnology[3]. The T-DNA is part of the ~200 kb Ti (tumour-inducing) plasmid, which also encodes functions for Ti plasmid conjugation, opine metabolism and the initiation, transfer and processing of the T-DNA[4,5]. Before the discovery of the Ti plasmid, gall-inducing ability was shown to be transferable to non-virulent Agrobacteria and to *Rhizobium leguminosarum*[6]. Ti plasmid transfer to *Rhizobium trifolii* and *Phyllobacterium myrsinacearum* resulted in strains that caused galls on some plants[7,8], but a *Sinorhizobium meliloti* strain containing a Ti plasmid was not tumorigenic[9]. Although these experiments showed that close relatives of *Agrobacterium* could harbour the Ti plasmid, no direct molecular evidence of gene transfer to plants by these bacteria was reported, leaving open the possibility that gall formation may have resulted from hormonal perturbations in the host plant unrelated to DNA transfer[10]. Indeed, a disarmed Ti plasmid and binary vector were introduced into a bacterial isolate apparently related to *Phyllobacterium* spp. for the purpose of tobacco inoculation[11], and although galls resulted from production of auxin by *Phyllobacterium*, these galls were morphologically different from those produced by an *Agrobacterium*-transformed plant through gene transfer; moreover, evidence of gene transfer was sought but not found. Accordingly, the scientific community has focused on *Agrobacterium* as a vehicle for gene transfer; the vast majority of patent claims regarding biological plant transformation explicitly refer to *Agrobacterium*[2]. A recent proposal suggesting that *A. tumefaciens* be reclassified as *Rhizobium radiobacter* has been widely disputed[12], although *Agrobacterium* is clearly closely related to *Rhizobium*. However there is little doubt that *Agrobacterium*, *Sinorhizobium* and *Mesorhizobium* are in distinct phylogenetic clades and their genomic organization differs considerably[4,13].

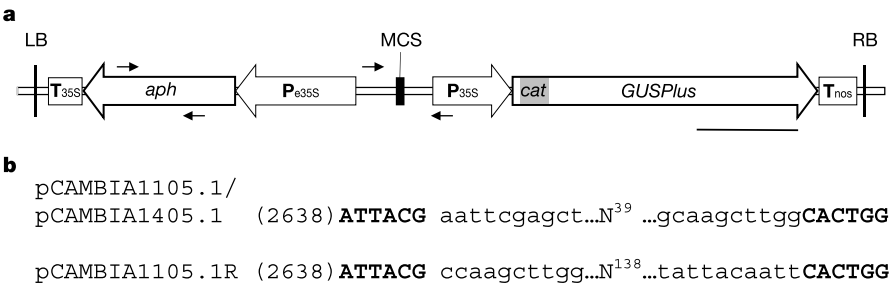

Figure 1 Binary vectors used for plant transformation. **a**, T-DNA map for pCAMBIA vectors used, containing the hygromycin resistance gene (*aph*) for plant selection and the *GUSPlus* gene as a reporter to investigate gene transfer. P35S and Pe35S, promoter and enhancer + promoter from CaMV35S, respectively; Tnos and T35S, terminators from *A. tumefaciens* nopaline synthase gene and CaMV35S, respectively; cat, catalase intron; LB and RB, left and right T-DNA border sequence. Arrows show the position of primers used for analysis of T-DNA integration; bar indicates position of probe used for Southern blot analysis. **b**, Multiple cloning site sequences in pCAMBIA1105.1 and pCAMBIA1405.1 (used in *A. tumefaciens*) and pCAMBIA1105.1R (exclusively used in non-*Agrobacterium* bacterial species).

To see whether non-Agrobacterial species could be made competent for gene transfer to plants, we introduced the disarmed Ti plasmid (pEHA105) from a hypervirulent *Agrobacterium* strain[14] into several different species of bacteria. To facilitate transfer of this large plasmid, we integrated the origin of transfer (*oriT*) of a broad-host range IncP plasmid into the Ti plasmid of EHA105 at two different locations (pTiWB1, pTiWB3). We then mobilized the modified Ti plasmids into *Rhizobium* sp. NGR234, an exceptionally broad-host range *Rhizobium* species that nodulates over 100 different plants[15]; the alfalfa symbiont *Sinorhizobium meliloti*; and *Mesorhizobium loti*, a representative of a different family (Phyllobacteriaceae). To assay gene transfer to plants, we introduced the binary vector pCAMBIA1105.1R into the non-*Agrobacterium* bacteria (Fig. 1a) and pCAMBIA1105.1 or pCAMBIA1405.1 into *Agrobacterium*. To confirm the provenance of insertion events, these vectors were constructed with unique T-DNA 'tags' giving rise to polymerase chain reaction (PCR) products of different size. The 'R' version (pCAMBIA1105.1R) was reserved for use only in rhizobia (Fig. 1b) and was never introduced into *Agrobacterium*. We developed additional primers to check the genotype of engineered strains by PCR (Fig. 2) and confirmed that the strains were free of contaminating *Agrobacterium* by selective plating.

Gene transfer to tobacco was investigated by co-cultivating leaf discs with bacterial strains and assaying for β-glucuronidase (GUS) activity (Fig. 3). *GUSPlus*, a synthetic *gusA* gene based on the sequence from *Staphylococcus* sp., produces a GUS protein detectable at up to tenfold higher sensitivity compared to *E. coli* GUS[16]. The intron inserted into the *GUSPlus* coding sequence prevents expression in bacterial cells, thus restricting activity to plant cells that had incorporated and expressed the transgene. All strains tested gave rise to GUS-expressing foci on the leaves and leaf margins, but these foci varied considerably in number and location (Fig. 3a). GUS activity was not observed in control experiments using *S. meliloti* or *Rhizobium* sp. that carried the binary vector but no Ti plasmid, suggesting that gene transfer was mediated through the *vir* gene machinery of pTiWB1 or pTiWB3. The number of GUS-expressing foci observed for tobacco following co-cultivation with various bacterial strains is compiled in Table 1. For non-*Agrobacterium* strains, the developmental stage of the leaves affected gene transfer considerably, with young leaves yielding an average of >10 times more GUS-expressing foci compared with older, just fully expanded leaves over several experiments. No difference was observed between the two leaf stages for *Agrobacterium* transformation. Longer co-culture times also increased gene transfer from non-*Agrobacterium* strains; this is perhaps associated with their slower growth.

To confirm that the transferred T-DNA integrated into the genome, plants were regenerated from transformation events using three non-*Agrobacterium* species (Table 2). Regenerated plants showed GUS activity in their leaves (Fig. 3b), and PCR amplification of the 'provenance-tagged' site confirmed that the T-DNA originated from the binary vector exclusively introduced into non-*Agrobacterium* strains (Fig. 3c). Eleven plants were analysed by Southern blotting, showing stable integration of T-DNA at 1–3 sites per plant (Fig. 3d). Sequences of T-DNA insertion site(s) were determined for several plants, revealing integration into independent loci (Fig. 3e), some of them matching known tobacco sequences. This suggests that a number of diverse plant-associated bacteria, when harbouring a Ti plasmid and binary vector, are able to transfer T-DNA to plants.

The bacterial strains were also tested for their ability to transform other plant species. Rice calli that were co-cultivated with *S. meliloti* (containing pTiWB3 and pCAMBIA1405.1) revealed GUS activity in four out of 687 calli (0.6%). Subsequently, using pTiWB3 and

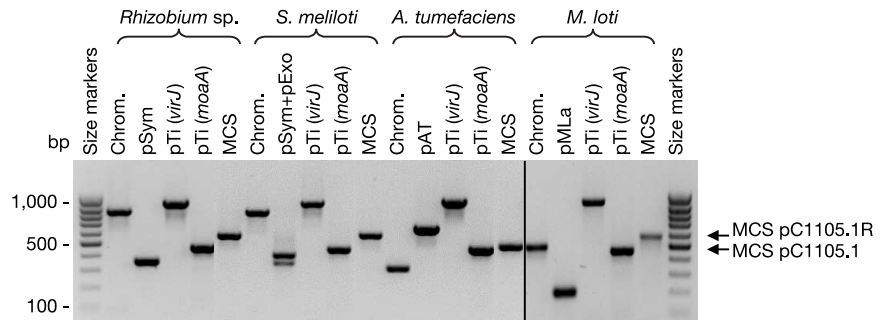

Figure 2 Genotyping of gene transfer-competent bacterial strains containing pTiWB3 by selective PCR amplification of chromosomal and plasmid markers (see Supplementary Table S1), and the differently sized MCS in pCAMBIA1105.1R (pC1105.1R) and pCAMBIA1105.1 (pC1105.1) vectors (arrows).

pCAMBIA1105.1R, one transformed rice plant (from a total of 695 calli) regenerated and rooted (Fig. 3b, c). Analysis of the single T-DNA integration site in this rice plant (Fig. 3d) indicated that the T-DNA had integrated into rice chromosome 11 (BAC clone OSJNBa0081F16; Fig. 3e). In contrast, in a typical rice transformation experiment using *Agrobacterium*, 50–80% of rice calli co-cultivated for 2 days show GUS-expressing foci and 20% of all calli regenerate transformed shoots.

Arabidopsis transformation[17] following floral dip in a *S. meliloti* suspension resulted in six hygromycin-resistant seedlings from approximately 70,000 seeds, representing 5–10% of the efficiency of *A. tumefaciens* using the same protocol. One plant was also obtained from *Rhizobium*-mediated transformation of *Arabidopsis*. All seven plants expressed GUS in their leaves (Fig. 3b) and contained the T-DNA from pCAMBIA1105.1R (Fig. 3c). Southern blot analysis of two of the plants transformed by *S. meliloti* indicated T-DNA integration at one and three genomic loci respectively (Fig. 3d). The single T-DNA insertion in plant number 4 showed a perfect match to the *A. thaliana* protein phosphatase 2C gene on chromosome I (Fig. 3e). Sequencing of the T-DNA insertion site for plant 5 showed a match at the left and right border with a site in *Arabidopsis* chromosome I (BAC clones T23G18 and T6D22), with a 20 bp deletion at the insertion site. The T-DNA insertion site for plant 6 was on chromosome III (*Arabidopsis* BAC clone F16B3). In a subsequent floral dip experiment with *S. meliloti* in which the infiltration medium was modified, two transformed plants were obtained from approximately 4,000 seeds, a fourfold improvement over previous conditions.

These results confirm that all three non-*Agrobacterium* species

Table 1 **Gene transfer to tobacco by diverse species of bacteria**

Species	Ti plasmid*	Number of leaf discs† assayed (number of experiments)	Average number of GUS foci per leaf disc ± s.d.‡
S. meliloti	pTiWB1	40 (4)	49 ± 50
	pTiWB3	69 (7)	53 ± 55
Rhizobium sp. NGR234	pTiWB1	40 (4)	9 ± 12
	pTiWB3	59 (6)	8 ± 15
M. loti	pTiWB1	20 (2)	3 ± 5

*In these experiments, the Ti plasmids are not being compared for gene transfer efficiency as they were not always assayed in parallel. In *Agrobacterium*, similar tobacco transformation efficiencies were observed with either the wild-type Ti plasmid or pTiWB1 or pTiWB3 (data not shown).
†For the data in this table, leaf discs were cut from young (not fully expanded) leaves.
‡Similar experiments with *A. tumefaciens* yielded a typical transformation efficiency of 200–400 GUS foci per leaf disc.

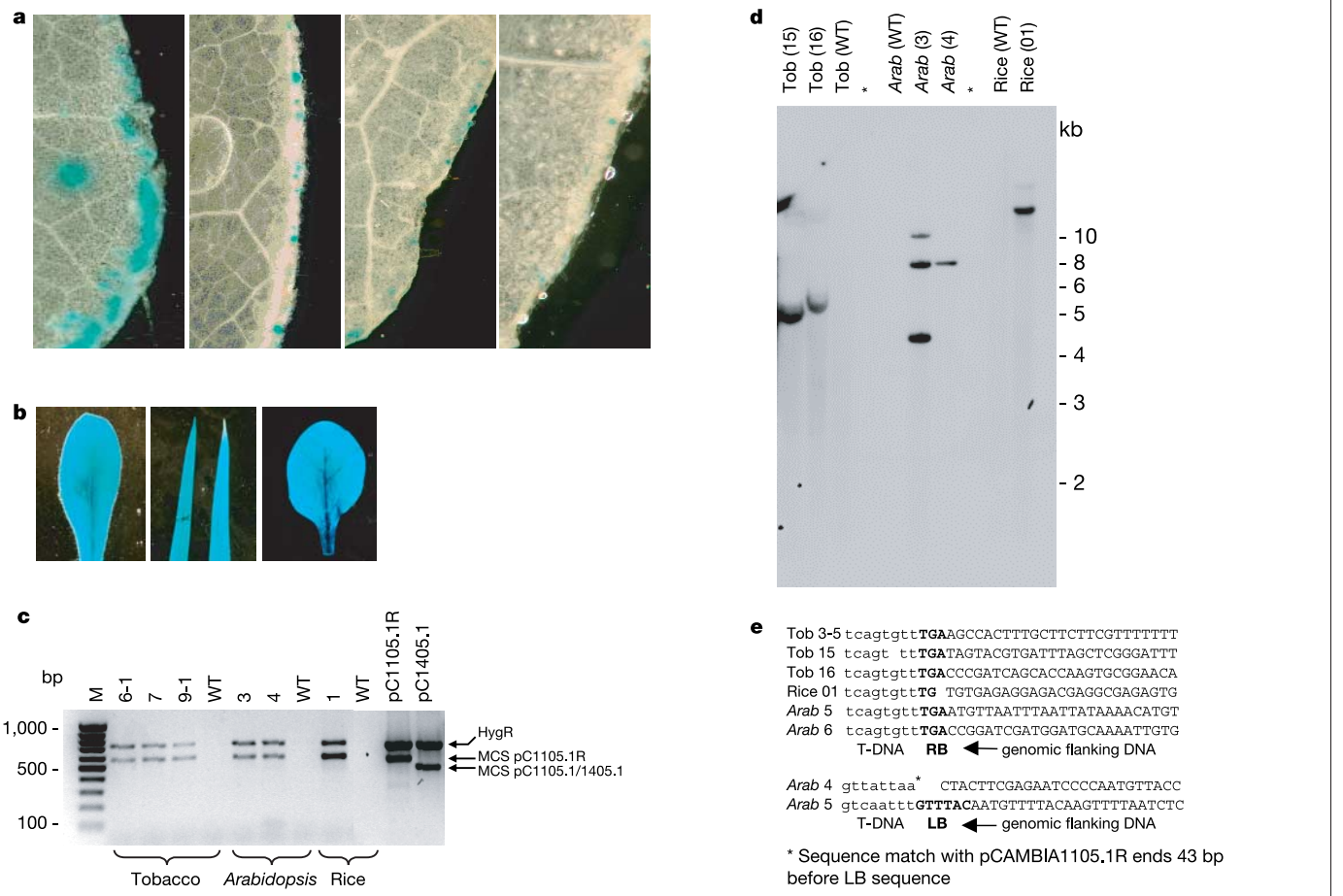

Figure 3 Gene transfer to plants by diverse species of bacteria. **a**, Tobacco leaf discs stained for GUS activity immediately after co-cultivation with (left to right) *A. tumefaciens*, *Rhizobium* sp. NGR234, *S. meliloti* or *M. loti*. **b**, GUS activity in leaves of tobacco (left), rice (middle) and *Arabidopsis* (right), regenerated following transformation with *Rhizobium* sp. NGR234 (tobacco) or *S. meliloti* (rice and *Arabidopsis*). **c**, Duplex PCR analysis for the hygromycin resistance gene (HygR, upper band) and the T-DNA MCS (lower band) in plants transformed through *Rhizobium* sp. NGR234 (tobacco) or *S. meliloti* (*Arabidopsis* and rice). WT, untransformed plant; plasmid controls pCAMBIA1105.1R (pC1105.1R), pCAMBIA1405.1 (pC1405.1); numbers refer to different transformants. **d**, Southern blot analysis of transgenic tobacco (Tob), *Arabidopsis* (*Arab*) and rice plants and their respective untransformed controls (WT). Plant number indicated in parentheses. Transgenic plants shown here resulted from *S. meliloti*–mediated transformation. Asterisks used to denote an empty lane. **e**, Sequences of the T-DNA RB insertion site in three tobacco plants and a rice plant, and of the RB or LB insertion sites in three *Arabidopsis* plants after *S. meliloti*-mediated transformation. Lower case letters indicate sequence of T-DNA; bold letters indicate the border sequence of the T-DNA (RB, LB); upper case letters indicate flanking DNA sequences.

Table 2 **Recovery of transgenic tobacco plants following gene transfer mediated by bacteria**

Bacterial strain	Number of leaf discs (number of experiments)	Number of transformed plants*	Transformation frequency† (%)
S. meliloti pTiWB3	116 (7)	33	28
S. meliloti pTiWB1	22 (2)	8	36
Rhizobium sp. pTiWB3	148 (11)	13	9
M. loti pTiWB1	30 (3)	4‡	13

*T-DNA confirmed by PCR.
†Number of transformed plants obtained per 100 leaf discs. Similar experiments with *A. tumefaciens* yielded a typical transformation frequency of 95–100%.
‡Un-rooted shoots.

tested here can transform plants. Of these, at least *S. meliloti* is competent to transfer genes into both dicot and monocot plants and into a range of tissues, including leaf tissue, undifferentiated calli and immature ovules.

Genes not resident on the Ti plasmid, but located on the *Agrobacterium* chromosomes, have been described as essential for virulence[1]. Gene transfer is, however, only a component of the more complex virulence phenotype, and may not require these additional loci. Our observations suggest that if there are gene functions necessary for gene transfer that are not encoded by the Ti plasmid, they must have equivalents or homologues[13] in multiple rhizobial species. However it is possible that the small number of *vir*-related genes on the Ti plasmid are sufficient to confer gene transfer competence to any bacterium.

Our results suggest a mechanism by which natural, bacterially mediated horizontal gene transfer may have contributed to the composition of plant genomes. There are many genes of presumed bacterial origin in the genomes of plants and other eukaryotes[18–21]. In a simple case, an apparently ancient integration of T-DNA-derived *rol* genes into the wild-type tobacco genome has been described[22]. However, it is possible that more substantial gene transfer has occurred over an evolutionary timescale. This could have been mediated by Ti-encoded plant gene transfer or a similar conjugation pathway, but in a manner analogous to the Hfr phenomenon in bacterial mating[23] in which larger bacterial genome segments are transferred to recipient cells.

Many important crops, including species particularly relevant to the developing world, are difficult to transform by *Agrobacterium*, difficult to regenerate from a susceptible explant, or prone to unsuitable genetic and phenotypic variation using current methodology. *Agrobacterium* has evolved as a plant pathogen, presumably with constraints on its specific binding and infection characteristics, and with co-evolved plant responses to pathogenicity. Other plant-associated bacteria, including *Sinorhizobium*, *Rhizobium* and *Mesorhizobium*, interact with different cell types and stages, and as symbionts or benign endo- and epiphytes can be expected to evoke different plant responses. We may benefit from this broader range of interactions to facilitate gene transfer to previously intractable plant cell types, explants or species.

Optimization of this gene transfer technology may require changes to the genetics and/or the physiology of the host strain and the target plant species. Introducing *Agrobacterium* chromosomal genes into new bacterial species could enhance the induction or expression of *vir* genes[24]. Matching bacterial and plant genotypes or modifying culture conditions may also prove extremely effective. The tenfold enhancement in tobacco transformation using younger explants suggests many avenues for further improvements. Similarly, changes to the infiltration medium for *Arabidopsis* floral dip with *Sinorhizobium* increased the transformation frequency to almost half of the typical frequency using *Agrobacterium*, already making this a viable alternative technology.

We have developed gene transfer competent *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* strains, representing two different families of bacteria. This approach, based on acquisition of a disarmed Ti plasmid and a binary vector, is unlikely to be limited to these species of bacteria. In contrast to the complex licensing landscape for *Agrobacterium* methodology[2], this alternative technology is available to the international community in a 'protected technology commons' optimized and improved as a BioForge project (http://www.bioforge.net), and accessible under a BIOS (Biological Innovation for Open Society, http://www.bios.net) licence[25]. These open-source-modelled licences are characterized by having no commercial restrictions other than covenants for sharing of improvements, relevant safety information and regulatory data and for preserving the opportunity for others to freely improve and use the technology.

Note added in proof: Analysis of seed progeny of all three plant species transformed with *S. meliloti* shows stable inheritance of the transgenic GUS and hygromycin resistance phenotypes, with approximately mendelian segregation. □

Methods

Bacteria

Sinorhizobium meliloti 1021 and *Rhizobium* sp. NGR234 (streptomycin-resistant ANU240) were provided by the Australian National University Collection by M. Djordjevic; *Mesorhizobium loti* MAFF303099 was obtained from C. Ronson, and a single gentamicin phosphotransferase gene was inserted (this paper); *A. tumefaciens* EHA105 was obtained from P. Maliga. Bacteria were grown on YM plates or TY liquid with streptomycin (200 $\mu g\,ml^{-1}$; *S. meliloti*, *Rhizobium* sp.) or gentamicin (20 $\mu g\,ml^{-1}$; *M. loti*) except for *A. tumefaciens*, which was grown on LB.

The genotype of the bacterial strains used was confirmed by PCR amplification using selective primer sets for the chromosomes and plasmids. Details of genotyping primers used in Fig. 2 are listed in Supplementary Table S1; sequences of other primers that have been used in our work are available upon request.

Plasmid construction

For the creation of modified Ti plasmids, suicide vectors were constructed by T/A cloning of a PCR-amplified fragment from the Ti plasmid of EHA105 into TOPO vector pCR2.1 (Invitrogen), corresponding to 1,316 bp encompassing the whole *virG* gene (pTiWB1) or a 995 bp part of the non-essential gene *moaA* (pTiWB3). The *oriT* fragment of plasmid RK2/RP4 was cloned in these vectors as an *Xba*I fragment following amplification from pSUP202 using primers EVS49 and EVS50[26]. Electroporation of the suicide vectors into EHA105 resulted in integration of the whole vector by a single crossing-over event, thereby creating two functional *virG* genes (pTiWB1) or insertion of a second truncated *moaA* gene (pTiWB3). Co-integration was confirmed by PCR across the integration site and by Southern blotting showing duplication of the target locus.

The binary vector pCAMBIA1405.1 was derived from pCAMBIA1305.1 (GenBank accession number AF354045) by insertion of a spectinomycin/streptomycin marker into the *Sac*II site. To make pCAMBIA1105.1, the kanamycin marker in pCAMBIA1405.1 was removed and only the spectinomycin/streptomycin marker was retained. To create pCAMBIA1105.1R, the *Pvu*II-*Pvu*II multiple cloning site (MCS) fragment in pCAMBIA1105.1 was replaced by the larger *Pvu*II-*Pvu*II MCS from pCR2 after re-ligation of the *Eco*RI sites. The T-DNA region in these vectors was completely sequenced. Primers used to distinguish between pCAMBIA1105.1 ('non-R') and pCAMBIA1105.1R ('R') were 5′-CTGGCACGACAGGTTTC-3′ and 5′-TACGGCGAGTTCTGTTAGGT-3′, encompassing the MCS region.

Bacterial strain construction

Modified Ti plasmids were mobilized to *Sinorhizobium* and *Rhizobium* strains in a triparental mating with EHA105 containing either pTiWB1 or pTiWB3, an *E. coli* helper strain containing RP4-4, and the respective non-*Agrobacterium* acceptor strain. The pTiWB1 plasmid was mobilized to *M. loti* by mating with *Rhizobium* sp. NGR234 containing pTiWB1 and RP4-4. Colonies were then selected from transconjugants that did not contain RP4-4, as we discovered that its presence impaired plant transformation (data not shown). The binary vector pCAMBIA1105.1R was subsequently introduced by electroporation. Either pCAMBIA1105.1 or pCAMBIA1405.1 was electroporated into *A. tumefaciens* strain EHA105 containing either the unmodified pEHA105 Ti plasmid or pTiWB1 or pTiWB3.

Plant transformation

Bacteria were grown on plates, resuspended to an absorbance at 600 nm of 1.0–1.5 and used for transformation of tobacco leaf discs (*Nicotiana tabacum* L. cv. Wisconsin 38) following the protocol in ref. 27, except using RMOP media solidified with 2.5 $g\,l^{-1}$ Phytagel and a co-cultivation time of 3, 5 or 5–11 days (for *A. tumefaciens*, *S. meliloti* and *M. loti*, and *Rhizobium* sp., respectively). The bacterial cultures used for explant treatment were analysed by PCR with *Agrobacterium*-specific primer sets, and by plating onto LB plates favouring the growth of *Agrobacterium* in comparison with other rhizobia (a more sensitive assay). In 'addback' experiments (in which a defined number of *A. tumefaciens* cells were deliberately added to rhizobial cultures), this allowed detection of one *A. tumefaciens* cell in 10^9 rhizobial cells. This is several orders of magnitude more sensitive

than the PCR detection method. After co-culture, leaf discs were assayed for GUS activity (using 0.5 mg ml^{-1} X-glcA) or transferred to regeneration media containing antibiotics.

Rice (*Oryza sativa* L. cv. Millin) was transformed using seed-derived callus based on the protocol in ref. 28, with modifications (see Supplementary Information).

Arabidopsis thaliana L. cv. C24 was transformed using a floral-dip protocol[15] with bacteria resuspended in infiltration medium containing Murashige and Skoog Basal Medium, 5.0% (w/v) sucrose, 0.05% (v/v) Silwet L-77 and 0.1% (w/v) MES, pH 5.7. The modified media contained Murashige and Skoog Basal Medium, 1.0% (w/v) sucrose, 0.02% (v/v) Silwet L-77 and 0.1% (w/v) MES, pH 7.0.

Analysis of transformed plants

Genomic DNA was extracted from the regenerated plants using DNAzol (Invitrogen), digested with *Eco*RI and used for Southern blotting with a ^{32}P-labelled *GUSPlus* probe. Plant DNA sequences flanking the T-DNA insertion site(s) were determined by restriction digest/adaptor ligation[29].

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A universal trend of amino acid gain and loss in protein evolution

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Amino acid composition of proteins varies substantially between taxa and, thus, can evolve. For example, proteins from organisms with (G+C)-rich (or (A+T)-rich) genomes contain more (or fewer) amino acids encoded by (G+C)-rich codons[1–4]. However, no universal trends in ongoing changes of amino acid frequencies have been reported. We compared sets of orthologous proteins encoded by triplets of closely related genomes from 15 taxa representing all three domains of life (Bacteria, Archaea and Eukaryota), and used phylogenies to polarize amino acid substitutions. Cys, Met, His, Ser and Phe accrue in at least 14 taxa, whereas Pro, Ala, Glu and Gly are consistently lost. The same nine amino acids are currently accrued or lost in human proteins, as shown by analysis of non-synonymous single-nucleotide polymorphisms. All amino acids with declining frequencies are thought to be among the first incorporated into the genetic code; conversely, all amino acids with increasing frequencies, except Ser, were probably recruited late[5–7]. Thus, expansion of initially under-represented amino acids, which began over 3,400 million years ago[8,9], apparently continues to this day.

It is routinely assumed that extant proteins are in detailed equilibrium and their evolution is a stationary and reversible process: reciprocal fluxes of amino acid substitutions are equal, amino acid frequencies are constant, and nothing would change if time were to flow backwards[10–12]. Accordingly, symmetrical substitution matrices are used for protein sequence alignment[13].

We analysed 15 sets of three-way alignments of orthologous proteins encoded by triplets of closely related genomes (Table 1). At sites where the outgroup and one of the 'sister' genomes carry the same amino acid, whereas the other sister genome carries a different one, the amino acid ancestral for the sister genomes can be inferred. Accordingly, a specific, directed substitution can be assigned to the lineage of the sister that differs from the outgroup. For each set, we compared the 75 pairs of fluxes of forward and backward amino acid substitutions that can be caused by single-nucleotide replacements. The reciprocal fluxes differed by a factor of $\geq$1.5 for 575 of the 1,125 pairs (51%), and the differences were statistically signifi-