Syntenic Relationships between *Medicago truncatula* and Arabidopsis Reveal Extensive Divergence of Genome Organization[1][w]

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Arabidopsis and *Medicago truncatula* represent sister clades within the dicot subclass Rosidae. We used genetic map-based and bacterial artificial chromosome sequence-based approaches to estimate the level of synteny between the genomes of these model plant species. Mapping of 82 tentative orthologous gene pairs reveals a lack of extended macrosynteny between the two genomes, although marker collinearity is frequently observed over small genetic intervals. Divergence estimates based on non-synonymous nucleotide substitutions suggest that a majority of the genes under analysis have experienced duplication in Arabidopsis subsequent to divergence of the two genomes, potentially confounding synteny analysis. Moreover, in cases of localized synteny, genetically linked loci in *M. truncatula* often share multiple points of synteny with Arabidopsis; this latter observation is consistent with the large number of segmental duplications that compose the Arabidopsis genome. More detailed analysis, based on complete sequencing and annotation of three *M. truncatula* bacterial artificial chromosome contigs suggests that the two genomes are related by networks of microsynteny that are often highly degenerate. In some cases, the erosion of microsynteny could be ascribed to the selective gene loss from duplicated loci, whereas in other cases, it is due to the absence of close homologs of *M. truncatula* genes in Arabidopsis.

Comparative genetic mapping has revealed a high degree of conservation in genome structure among closely related plant species, in terms of gene content, order, and function (Paterson et al., 1995, 2000; Gale and Devos, 1998; Bennetzen, 2000). The best-documented cases of genome conservation are from the grass family (Poaceae), where rice (*Oryza sativa*) with its small genome has been selected as a nodal species to study the economically important cereal crops including corn (*Zea mays*), sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*; Devos and Gale, 2000). Extensive macrosynteny has also been observed within the Solanaceae (Tanksley et al., 1992), the Brassicaceae (Kowalski et al., 1994; Lagercrantz and Lydiate, 1996; Lagercrantz, 1998; O'Neill and Bancroft, 2000; Acarkan et al., 2000), and the Fabaceae (Weeden et al., 1992; Menacio-Hautea et al., 1993; Torres et al., 1993; H.-K. Choi and D.R. Cook, unpublished data).

In contrast to within-family comparisons, genome structure appears to be less conserved between distantly related species, where collinearity may only be apparent over small chromosomal intervals (Paterson et al., 1996, 2000). Paterson and colleagues have suggested that deciphering such relationships will require a very high density of genetic markers for comparison (Paterson et al., 2000). More recent analyses, however, reveal that plant genomes possess a dynamic microstructure (Ku et al., 2000; Vision et al., 2000) that may preclude establishing global relationships between distantly related plant species based on genetic map data alone. In lieu of whole-genome sequence data, an intermediate strategy involving complete sequencing and annotation of bacterial artificial chromosome (BAC)-size clones has been adopted by several groups to obtain a glimpse of genome similarities at the micro-level (Ku et al., 2000; Liu et al., 2001; Mayer et al., 2001; Rossberg et al., 2001). In the comparison of Arabidopsis and tomato (*Lycopersicon esculentum*), species that diverged early in the radiation of dicot plants, this strategy revealed an extensive network of microsynteny between a 105-kb segment of tomato chromosome 2 and Arabidopsis chromosomes 2 to 5 (Ku et al., 2000). It remains uncertain, however, whether such syntenic

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networks are a general feature of cross-family comparisons or only restricted to certain genome regions. Comparison of Arabidopsis and rice using a similar strategy revealed microsynteny at some loci but not others, leading to the conclusion that Arabidopsis may not be adequate as a model for the structure of grass genomes (Devos et al., 1999; van Dodeweerd et al., 1999; Liu et al., 2001; Mayer et al., 2001).

The Fabaceae, or legume species, make up the third largest family of flowering plants, including numerous important agricultural crops such as soybean (*Glycine max*), pea (*Pisum sativum*), beans (*Phaseolus vulgaris*), and alfalfa (*Medicago sativa*). Legumes are also distinguished by their unique property of symbiotic nitrogen fixation, providing one of the major sources of available nitrogen in the biosphere. The agronomic and ecologic importance of this group of plants has provided incentive to undertake genome analyses in species such as *Medicago truncatula*, soybean, and *Lotus japonicus* (Cook, 1999). An important near-term goal for these efforts is to determine the extent to which the complete sequence of the Arabidopsis genome will have predictive value for the structure of legume genomes. Here, we report the results from a comparison of genome structure between the two model species *M. truncatula* and Arabidopsis. Our results suggest that the two genomes share in common an eroded network of microsynteny, but that broad macrosyntenic relationships are not apparent.

RESULTS AND DISCUSSION

High Level of Macrosynteny Is Not Evident between *M. truncatula* and Arabidopsis

As a prelude to comparative genome analysis between *M. truncatula* and Arabidopsis, we analyzed approximately 300 mapped genes in *M. truncatula* to identify their highly conserved counterparts in Arabidopsis (see criteria in "Materials and Methods" and specific comparisons in online supplemental data, which can be viewed at www.plantphysiol.org). The rationale for focusing on highly conserved genes is as follows. We expect that conserved gene order will be most evident in cases where linkage is derived from the most recent common ancestor. By definition, such genes are orthologous between the species under comparison. A corollary to this logic is that poorly conserved genes will be of limited value to identify ancestral synteny, in particular because such genes are likely to represent ancient paralogs derived through a process of within-species duplication. Our approach contrasts with that of previous studies to compare a legume genome with that of Arabidopsis (Grant et al., 2000), where low similarity criteria were used to examine conserved gene order between soybean and Arabidopsis.

One hundred and ten of the mapped *M. truncatula* genes possess significant homology to Arabidopsis genes according to the criteria described in "Materials and Methods" and presented in the supplemental data. Thirty of these *M. truncatula* genes had only a single match in Arabidopsis, whereas the remaining 80 (approximately 73%) had two or more matches. This redundancy of homology is consistent with the duplicated nature of the Arabidopsis genome (Arabidopsis Genome Initiative, 2000).

Alignment of the *M. truncatula* and Arabidopsis chromosomes reveals a complex relationship between the two genomes (Fig. 1). In particular, many mapped *M. truncatula* genes share high homology to several loci within the Arabidopsis genome. As a consequence, each of the eight *M. truncatula* linkage groups can be aligned to any of the five Arabidopsis chromosomes. In an effort to obtain a "one-to-one" comparison between the two genomes, we focused

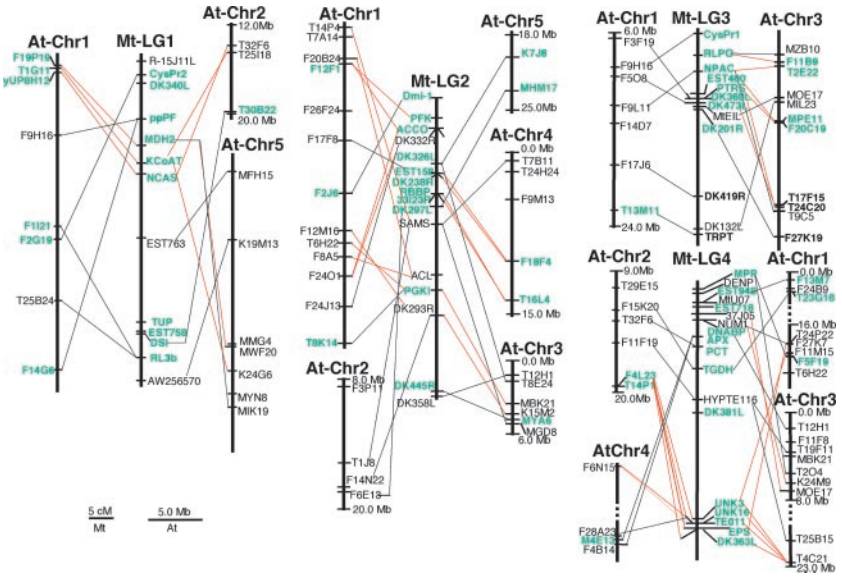


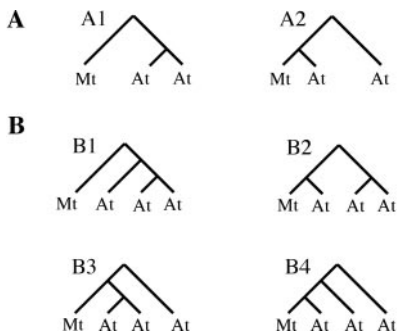
Figure 1. Chromosomal alignments between the *M. truncatula* genetic linkage groups 1 to 4 and the Arabidopsis chromosomes. Homologs between the two genomes are connected with lines. The markers with green color are putative orthologous loci. Red lines indicate the possible syntenic relationships between the two genomes.

on tentative orthologous pairs of loci gained from the eukaryotic gene orthologs database of The Institute for Genomic Research (TIGR; http://www.tigr.org/tdb/tgi/ego/index.shtml). On the basis of this analysis, 82 pairs of loci were found to be tentative orthologs (see supplemental data). Comparing linkage relationships between tentative orthologs did not reveal a simplified picture of synteny between the *M. truncatula* and Arabidopsis genomes. In most cases, the Arabidopsis orthologs of linked *M. truncatula* genes are either not linked in Arabidopsis or linked but interrupted by orthologs that are not linked in *M. truncatula* (Fig. 1). This result is in contrast to that reported by Grant et al. (2000), where significant synteny was reported between soybean and Arabidopsis along the entire length of Arabidopsis chromosome 1 and soybean linkage group A2.

The convoluted relationship suggested by marker alignment can be explained by either the absence of extended synteny between the two genomes or synteny that is obscured by the duplication of individual genes or chromosomal segments followed by extensive reshuffling and selective gene loss in both genomes (Blanc et al., 2000; Ku et al., 2000). In cases of gene duplication, paralogs arising subsequent to separation of the *M. truncatula* and Arabidopsis lineages would be most complicating to synteny analysis because they increase the number of phylogenetically equidistant loci between the genomes. The TIGR ortholog prediction, used above, identifies the most conserved genes between genomes without regard to lineage relationship. As a counterpoint to the analysis of "orthologs" predicted by the eukaryotic gene ortholog analysis, we sought to predict the lineage relationships for all genes under comparison in Figure 1. Ratios of non-synonymous to synonymous nucleotide substitutions were similar within gene families and were substantially less than one for all gene pairs under analysis (data not shown). These results suggest both a high level of purifying selection and similar rates of evolution between paralogs. Thus, we used the rate of non-synonymous nucleotide substitution (K_a) as a measure of divergence for each conserved gene pair between *M. truncatula* and Arabidopsis, and between different homologs within the Arabidopsis genome (Table I). The average K_a for single-copy Arabidopsis genes compared with the corresponding marker gene in *M. truncatula* was 0.19 ± 0.09, with a range of 0.03 to 0.44. Similar aggregate values were obtained when K_a was calculated for *M. truncatula* genes with two or three homologs in Arabidopsis (i.e. for two homologs, $K_a = 0.18 \pm 0.12$ with a range of 0.03–0.45; for three homologs, $K_a = 0.17 \pm 0.12$ with a range of 0.05–0.50).

Table I. *Predicted lineage patterns of comparative genes between M. truncatula and Arabidopsis*

M. truncatula Genes with Two Homologs in Arabidopsis					*M. truncatula* Genes with Three Homologs in Arabidopsis							
Marker	K_{a1} Mt-At1	K_{a2} Mt-At2	K_a At1-At2	Inferred Lineage	Marker	K_{a1} Mt-At1	K_{a2} Mt-At2	K_{a3} Mt-At3	K_a At1-At2	K_a At1-At3	K_a At2-At3	Inferred Lineage
DK326L	0.034	0.038	0.015	A1	MDH2	0.037	0.053	0.21	0.047	0.216	0.198	B3
RBBP	0.051	0.176	0.114	A2	KCOAT	0.097	0.109	0.17	0.065	0.189	0.189	B3
TRPT	0.069	ND	0.078	ND	ACCO	0.163	0.218	0.223	0.226	0.247	0.08	B2
TGDH	0.079	0.084	0.023	A1	ACL	0.077	0.077	0.149	0.026	0.142	0.134	B3
RL3b	0.087	0.108	0.098	A2	PGKI	0.119	0.114	0.067	0.057	0.111	0.112	B2
DK455	0.087	0.088	0.083	A1	43A3R	0.189	ND	ND	0.012	0.21	0.213	B1
DK363	0.093	0.141	0.047	A1	RLPO	0.103	0.107	0.098	0.013	0.107	0.112	B2
NCAS	0.093	0.108	0.054	A1	DNABP	0.155	0.133	0.22	0.121	0.263	0.235	B3
TUP	0.093	0.153	0.172	A2	RNAH	0.104	0.115	0.082	0.054	0.114	0.123	B2
AAT	0.097	0.122	0.044	A1	PDL	0.111	0.291	0.233	0.299	0.211	0.341	B4
PTRS	0.106	0.115	0.036	A1	ENOL	0.083	0.274	0.352	0.254	0.346	0.37	B4
ppPF	0.109	0.117	0.052	A1	EIF	0.083	0.121	0.062	0.073	0.092	0.121	B2
EPS	0.113	0.117	0.01	A1	SQEX	0.146	0.149	0.12	0.098	0.212	0.178	B2
PPGM	0.116	0.116	0.067	A1	UNK16	0.432	ND	ND	0.016	ND	ND	B3
PCT	0.121	0.144	0.224	A2	TE011	0.516	0.496	0.492	0.272	0.447	0.452	B3
COA	0.137	0.154	0.082	A1								
12NIL	0.143	0.161	0.161	A2								
PFK	0.151	0.179	0.203	A1								
DK351	0.182	0.271	0.197	A2								
EST718	0.201	0.291	0.232	A2								
EST158	0.204	0.211	0.199	A1								
PPDK	0.212	0.44	0.458	A2								
UNK7	0.215	0.28	0.182	A1								
DK419	0.224	0.3	0.235	A2								
MPP	0.26	0.269	0.096	A1								
LB1	0.366	0.548	0.455	A2								
CRS	0.411	0.487	0.18	A1								
GH1	0.442	0.447	0.302	A1								



The largely similar average K_a values reflect the fact that many of the duplicated Arabidopsis genes are approximately equally diverged from their *M. truncatula* counterpart. When between-species K_a values were compared with within-species K_a values, it was possible to infer lineage relationships for the different genes, as shown in Table I. Thus, 18 of the 28 marker genes with two homologs (lineage pattern A1) and seven of the 16 marker genes with three homologs (lineage patterns B1 and B3) are predicted to have arisen by duplication in Arabidopsis after separation of the *M. truncatula* and Arabidopsis lineages, whereas in other cases, the gene duplications in Arabidopsis are predicted to predate the speciation event. For the cases where duplication occurred after speciation, comparison of genome structure is complicated by the proliferation of multiple, phylogenetically equidistant loci. We suggest that in such cases a true ortholog cannot be defined between the genomes, and that the utility of the TIGR ortholog set is restricted to identifying the most highly conserved paralogs.

Conserved Gene Order over Small Genetic Intervals

In contrast to the apparent absence of extended synteny between *M. truncatula* and Arabidopsis, we observed frequent conservation of gene order over shorter genetic intervals. For example, three *M. truncatula* genes (Mt-MDH2, Mt-KcoAT, and Mt-NCAS) are within a 5-centiMorgan (cM) interval on the *M. truncatula* linkage group 1 (Mt-LG1), whereas their tentative orthologs (F19P19/At1g04410, T1G11/At1g04710, and yUP8H12/At1g05150, respectively) are in the same order and resident within a 400-kb region on the Arabidopsis chromosome 1 (At-Chr1; Fig. 1). More distant homologs of Mt-KcoAT and Mt-NCAS, namely F25I18/At2g33150 and T32F6/At2g32450, respectively, are also tightly linked within a 400-kb block on the At-Chr2. These two Arabidopsis regions have been previously described to be homeologous (Blanc et al., 2000), and the absence of a homolog of Mt-MDH2 from the At-Chr2 region is consistent with the finding that homeologous regions of Arabidopsis can be highly degenerate, with less than one-half of the gene homologs retained (Arabidopsis Genome Initiative, 2000).

In some cases, homologs of tightly linked genes on the *M. truncatula* genetic map are resident in the same BAC or BAC contig in Arabidopsis, providing further evidence of conserved local gene content. A typical example is from Mt-LG4, where a tight cluster of five genes (Mt-UNK3, Mt-UNK16, Mt-TE011, Mt-ESP, and Mt-DK363L) within a 2-cM interval are collinear with a BAC contig (At-T14P1 and At-F4L23) on At-Chr2 that contains all five of the predicted orthologs in Arabidopsis. Similar to the situation described above (Fig. 1), BAC contigs with less completely conserved gene content are distributed over two additional Arabidopsis chromosomes. Thus, BAC At-T4C21 on At-Chr3 and At-F6N15 on At-Chr4 contain four and two homologs of the five genes, respectively. Consistent with the apparent absence of extended macrosynteny, the observed local synteny does not appear to extend over longer genome intervals. For example, of four closely linked genes within a 4-cM interval on Mt-LG3 (i.e. Mt-EST400, Mt-PTRS, Mt-DK360L, and Mt-DK473L), the syntenic pair of Mt-EST400 and Mt-PTRS (MPE11/BAB01073 and F20C19/At3g26340, respectively) is only loosely linked to that of Mt-DK360L and Mt-DK473L (T24C20/At3g48190 and T17F15/At3g48000, respectively) on At-Chr3. A similar conservation of gene content and order has been observed between a small region of the soybean and Arabidopsis genomes (Foster-Hartnett et al., 2002).

Segmental duplication has been well documented in the Arabidopsis genome (Vision et al., 2000). Here we observe (a) that many close homologs between the *M. truncatula* and Arabidopsis genomes have undergone duplication subsequent to separation of the two lineages, and (b) that retained synteny is confined to small genome segments that are often repeated in the Arabidopsis genome. Thus, frequent segmental duplication and rearrangement after speciation are primary factors contributing to divergence between the *M. truncatula* and Arabidopsis genomes.

Microsynteny between *M. truncatula* and Arabidopsis at the BAC Clone Level

Two *M. truncatula* BAC contigs, Mt-AHC1 (AY224188) and Mt-AHC2 (AY224189), together with the BAC clone Mt-08D15 (AC087771) were subjected to complete sequencing. Mt-AHC1 and Mt-AHC2 were selected because they represent a segmental duplication in *M. truncatula*, initially identified based on the presence of highly conserved adenosylhomocysteinase (AHC) gene paralogs. The resulting sequences for Mt-AHC1, Mt-AHC2, and Mt-08D15 are approximately 100, 68, and 70 kb and contain 15, 12, and eight predicted genes, respectively. The average gene density of the three sequenced BACs is about one gene per 6.8 kb. Detailed analysis of these sequences and comparison with Arabidopsis are summarized in Table II and Figure 2.

Mt-AHC1 and Mt-AHC2 contain three pairs of homologous genes with the same order and orientation (i.e. Mt-AHC1.8 versus Mt-AHC2.3, Mt-AHC1.9 versus Mt-AHC2.4, and Mt-AHC1.14 versus Mt-AHC2.9; Table II). The three genes span 57 kb in Mt-AHC1 but only 40 kb in Mt-AHC2 (Fig. 2A). The increased physical interval in Mt-AHC1 is primarily due to longer introns in Mt-AHC1 genes rather than to extended intergenic regions. Comparing *M. truncatula* genes on Mt-AHC1 and Mt-AHC2 with their counterparts in Arabidopsis reveals a network of synteny (Fig. 2A), similar to that observed between

Table II. *Matches of predicted M. truncatula genes with Arabidopsis*[a]

Predicted Open Reading Frames	Expressed Sequence Tag (EST) Matches[b]	Matches of Arabidopsis Genes					Putative Function
		At-chr1	At-chr2	At-chr3	At-chr4	At-chr5	
AHC1.8	TC25520	At1g36370			At4g13890, At4g13930	At5g26780	
		At1g22020			At4g32520, At4g37930		Hydroxymethyltransferase
AHC1.9	TC21970			AT3g23810	At4g13940		Adenosylhomocysteinase
AHC1.10	TC16577				At4g13980		Heat shock transcription factor
AHC1.11	NA				At4g21110		G10-like protein
AHC1.12	NA			A large gene family			Hypothetical protein
AHC1.13	TC18388				At4g08580	At5g17900	Microfibril-associated protein
AHC1.14	AL383790			At3g23800	At4g14030, At4g14040		Selenium-binding protein
AHC1.15	NA			At3g23790	At4g14070		AMP-binding protein
AHC2.1	BE999775				At4g13870		Unknown protein
AHC2.2	AW736328		At2g31970				RAD50 DNA repair protein
AHC2.3	TC22017			Same as those of AHC1.8			Hydroxymethyltransferase
AHC2.4	TC21843			At3g23810	At4g13940		Adenosylhomocysteinase
AHC2.5	NA			A large gene family			Polygalacturonase
AHC2.6	NA						No match
AHC2.7	NA			A large gene family			Polygalacturonase
AHC2.8	NA		At2g31990		At4g13990		Hypothetical protein
AHC2.9	TC19298			At3g23800	At4g14030, At4g14040		Selenium-binding protein
AHC2.10	NA		At2g25010				Unknown protein
AHC2.11	NA						No significant match
AHC2.12	NA				At4g14050		Hypothetical protein
08D15.1	TC22974	At1g67950		At3g01210	At4g17720	At5g16840	
						At5g46870	Hypothetical protein
08D15.2	TC17359	At1g32270			At4g17730	At5g16830	
						At5g46860	Syntaxin
08D15.3	AW585392	At1g32310					Unknown protein
08D15.4	TC17232	At1g32330		At3g02990	At4g17750	At5g16820	Heat shock transcription factor HSF1
08D15.5	NA						No significant match
08D15.6	BE247890	At1g32340					RING finger protein
08D15.7	NA						No significant match
08D15.8	NA	At1g10680			At4g25960		P-Glycoprotein-2

[a] For genes with multiple hits, only representatives are listed; for AHC1 locus, only genes that are within the syntenic range are listed. [b] NA, EST match not available.

tomato and Arabidopsis (Ku et al., 2000). Gene order is conserved among syntenic blocks, with a reversal of orientation observed only in two cases (Mt-AHC2.12 versus FCA0/At4g14050 and FCA0/At4g13990 versus F22D22/At2g31990). The BAC contig made up of At-F18A5 and At-FCA0 on At-Chr4 contains two cases of gene duplication: One is a tandem duplication (FCA0/At4g14030 and FCA0/At4g14040), whereas the other (F18A5/At4g13890 and FCA0/At4g13930) is a direct duplication separated by three *cf*-like disease resistance gene homologs (see below). Interestingly, this At-Chr4 BAC contig appears to be most representative of the ancestral chromosomal segment from which the other syntenic blocks are predicted to have originated, because it contains all but one (Mt-AHC2.2) of the syntenic genes observed both in *M. truncatula* and Arabidopsis. The less complete syntenic blocks on At-MYM9 and At-F22D22 in Arabidopsis probably arose from segmental duplication followed by gene deletion events (Ku et al., 2000).

Similar results were obtained when comparing genes on *M. truncatula* BAC clone Mt-08D15 with their homologs in Arabidopsis (Fig. 2B). Five of the eight predicted genes from Mt-08D15 (Mt-08D15.1, Mt-08D15.2, Mt-08D15.3, Mt-08D15.4, and Mt-08D15.6) can be aligned with genes on four different Arabidopsis BAC clones (At-F5D14, At-F5E19, At-FCA9, and At-MSD23). All syntenic genes are in the same order and orientation, and the intergenic spaces and intron sequences are generally longer in *M. truncatula* than in Arabidopsis.

Microsynteny between BAC Clones Is Highly Degenerate

The 42 syntenic genes identified in the AHC and 08D15 contigs correspond to 14 unique gene homologies that are shared by at least one of the syntenic loci in both Arabidopsis and *M. truncatula* (Fig. 2). In the case of the AHC contigs, nine homologs are predicted to define the minimal structure of the ancestral locus (Fig. 2A, letters A–I). Interestingly, none of the conserved homologs were conserved in all of the duplicated regions, suggesting that the structure of the ancestral locus is degenerate in all of these modern day descendants. In cases where absent homologs are flanked by a conserved framework (e.g. homolog E in Mt-AHC2 and homolog F in Mt-AHC1), selective gene loss after duplication is a likely cause of degenerate microsynteny (Ku et al., 2000). At the extremity of syntenic regions, the absence of conserved homologs is indicative of either (a) small size of the segmental duplication, or (b) degenerate synteny that extends beyond the sequenced region in *M. truncatula*. Alignment of the structures of the AHC loci from Arabidopsis and *M. truncatula* (Fig. 3) indicates that each of the five loci

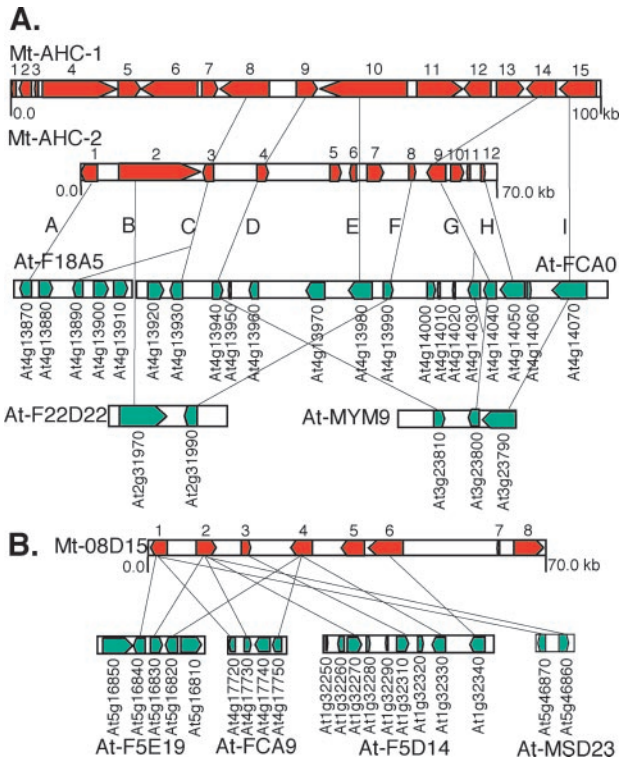


Figure 2. Microsynteny between sequenced *M. truncatula* BACs and segments of Arabidopsis chromosomes. The orientations of predicted genes are indicated by arrows. Letters A through I in A represent the nine unique gene homologies found between Arabidopsis and *M. truncatula* and thus define the minimum structure of the ancestral chromosome segment from which all the syntenic blocks were derived. A detailed description of gene annotations and homologies is given in Table II. The maps are drawn to scale.

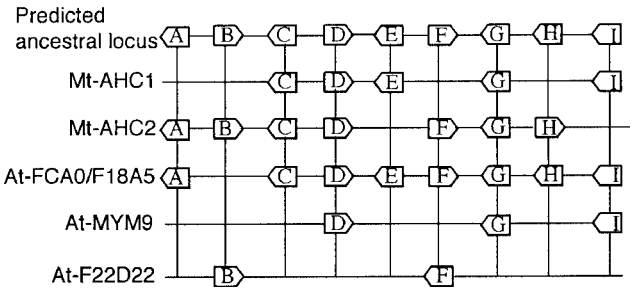


Figure 3. Reconstruction of the predicted ancestral AHC locus representing five segments from the *M. truncatula* and Arabidopsis genomes. The transcriptional orientations of the genes are indicated by arrows. Letters A through I are consistent with those in Figure 2.

(two in *M. truncatula* and three in Arabidopsis) has experienced selective gene loss. If the loss of adjacent genes is considered to be a single event, then the overall rate of gene loss for *M. truncatula* and Arabidopsis are similar (i.e. five events per 18 genes [28%] and seven events per 27 genes [26%], respectively).

In some cases, non-syntenic genes within a conserved framework are members of large gene families. For example, the syntenic segment on At-Chr4 contains a cluster of four *cf*-like disease resistance genes (F18A5/At4g13880, F18A5/At4g13900, F18A5/At4g13910, and FCA0/At4g13920) within a 20-kb interval, three of which are flanked by two highly similar hydroxymethyltransferase (HMT) genes (F18A5/At4g13890 and FCA0/At4g13930; Fig. 2A). This nested duplication structure is reminiscent of the tomato *cf-4/9* locus, where the *cf* resistance gene homologs are flanked by two conserved lipoxygenase genes (Parniske et al., 1997). Such genomic microstructure may provide substrates for unequal crossing-over events and thus contribute to expansion or contraction of local gene redundancy. In the case of resistance gene homologs, unequal crossing-over is implicated in the fast-evolving nature of these genes, which are often poorly conserved even between closely related genomes (Leister et al., 1998). FCA0/At4g13960, a member of F-box gene family, is also not represented in the *M. truncatula* AHC syntenic regions.

We also observed interspersion of low copy genes between syntenic genes both within and between *M. truncatula* and Arabidopsis (Fig. 2). This might be due to the loss of different subsets of genes from a common ancestor, independent gain of genes through transposition events, or reshuffling of small DNA segments (Blanc et al., 2000; Ku et al., 2000; Bancroft, 2001).

Divergence Estimates and Rearrangements to Gene Structure within Segmental Duplications

To understand the evolution of duplicated genes within and between species, we compared gene structures and phylogeny of the three genes present in both members of the *M. truncatula* AHC segmental

duplication (i.e. AHC, HMT, and selenium-binding protein [SBP]; Table I). In the Arabidopsis genome, highly similar homologs of SBP and AHC are confined to the segmental duplications shown in Figure 2. The HMT gene family, on the other hand, is represented by several additional homologs elsewhere in the Arabidopsis genome. To determine the probable order of speciation versus segmental duplication, we conducted phylogenetic analysis by means of the neighbor-joining method. The resulting tree topologies, which are supported by 100% of 1,000 bootstrap replications in all cases of syntenic homologs, suggest that these segmental duplications derive from independent events that occurred subsequent to separation of the *M. truncatula* and Arabidopsis lineages (Fig. 4).

As a further measure of diversification, we compared intron-exon structures for the three genes described above (Fig. 4). As shown diagrammatically in Figure 4, the position and number of introns is largely conserved, whereas intron lengths are variable both within and between *M. truncatula* and Arabidopsis, with intron length generally longer in *M. truncatula*. Despite the overall conservation of gene structure, intron loss is evident for some of these duplicated genes. For example, the HMT gene At4g13890, which is a close paralog of At4g13930 on At-Chr4, is predicted to have lost the first intron by fusion of the first and second exons from the At4g13930 (Fig. 4A). The SBP gene At4g14040, a tandem duplication of At4g14030, is similarly missing the third intron (Fig. 4C), whereas SBP Mt-AHC1.14 exhibits a truncated N terminus and an enlarged second intron. Despite this fact, the Mt-AHC1.14 represents an expressed gene, as evidenced by a matching *M. truncatula* EST in the *M. truncatula* gene index (MtGI) database (AL383790).

In the case of the HMT gene family, the intron-exon structures of syntenic compared with non-syntenic homologs are highly diverged. Phylogenetic analysis of HMT genes (Fig. 4A) indicates that the syntenic and non-syntenic homologs belong to separate lineages, with similar intron-exon structures within a lineage but substantially different intron-exon structures between lineages. Because these HMT homologs have a generally conserved global alignment, we suggest that exon splicing has contributed to the differences in intron/exon number and has thus played a role in the divergence of this small gene family. In other instances, exon shuffling is implicated as a mechanism of divergence; for example, two HMT homologous genes, At1g22020 and At1g36370, are distinguished by a approximately 120-amino acid domain of unknown origin at their N terminus. The existence of *M. truncatula* HMT genes (e.g. TC22007) with a similar predicted intron-exon structure and amino acid sequence to those found in Arabidopsis (i.e. At4g37930 and At5g26780) demonstrates that this divergence is likely to predate separation of the *M. truncatula* and Arabidopsis lineages.

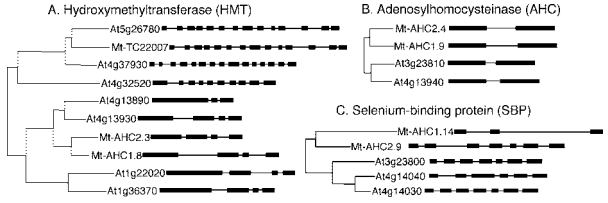


Figure 4. Phylogeny versus gene structure of syntenic and non-syntenic homologous genes. Phylogenetic analyses of protein sequences were performed using the ClustalX program (Thompson et al., 1997), and trees were constructed using the neighbor-joining method. The intron-exon structures of genes are drawn to scale. Exons are shown as boxes and introns are shown as lines. Intron-exon structures were inferred based on comparison of EST and genomic sequence data and based on gene prediction tools, as described in "Materials and Methods." The predicted structure of Mt-TC22007 is from TIGR database, and the intron sizes are unknown.

Frequency of Microsynteny between *M. truncatula* and Arabidopsis

An important question regarding microsynteny between distantly related species is whether the microsynteny observed in case studies of completely sequenced BAC clones is indicative of a wider conservation of genome structure. To address this issue, we analyzed 40 *M. truncatula* BAC clones/contigs that were subject to either survey sequencing or lower coverage sequence analysis based on a combination of BAC end and targeted internal sequence analysis. The short gene sequences obtained from BAC survey sequencing can be easily anchored to longer tentative consensus sequences (TCs) by means of querying MtGI using BLASTN, thus permitting a better informed analysis of conserved microsynteny between *M. truncatula* and Arabidopsis. The existence of microsynteny was inferred when close homologs of genes identified on a *M. truncatula* BAC (or contig) were also resident on a single Arabidopsis BAC or adjacent BACs. Through this analysis, 17 of the 40 loci show some level of microsynteny with one or more regions in Arabidopsis (see supplemental data), underscoring the conclusion that the genomes of *M. truncatula* and Arabidopsis are related by numerous regions of degenerate microsynteny.

The Potential Role of Species-Specific Genes in Genome Divergence

BAC survey sequencing indicates that the erosion of microsynteny is in part due to the absence of close homologs of some *M. truncatula* genes in Arabidopsis. For example, BAC end sequences from a *M. truncatula* BAC contig (BAC clones 21E21, 21L09, 33P03, and 79M20) identified three genes: *nodulin25* (AJ277858), *MtN22* (CAA75576; Gamas et al., 1996), and *TC23093*. A search of the *Medicago truncatula* GeneIndex (www.tigr.org/tdg/mtgi) reveals that all

three genes are expressed to a higher level, exclusively in root nodules. *Nodulin25* and *MtN22* genes have no close homologs in Arabidopsis, while TC23093 is a member of a calmodulin multigene family. In addition to contributing to divergent genome structure, genes with taxonomically restricted homologies are prime candidates for important roles in determining lineage-specific phenotypes, such as symbiotic nitrogen fixation in legumes. A specific example of this situation is the case of the nodulation receptor kinase (NORK) locus (*dmi2* in *M. truncatula*) that was recently cloned in *M. truncatula*, alfalfa, pea, and *L. japonicus* based on their conserved syntenic positions (Endre et al., 2002; Stracke et al., 2002). The NORK protein is a putative receptor kinase that is highly conserved among all legume species analyzed (Endre et al., 2002). Although the genome region that contains *dmi2* is microsyntenic between *M. truncatula* and Arabidopsis, the NORK homolog is missing from the syntenic region of Arabidopsis, and only very distant homologs are evident elsewhere in the genome.

Summary

Comparative genome analysis between the model legume *M. truncatula* and Arabidopsis reveals a lack of extensive macrosynteny between these two genomes, whereas genetic evidence as well as both complete and low coverage sequencing of *M. truncatula* BAC clones support a model of degenerate microsynteny. Our data suggest that diversification between these two genomes is driven by at least three factors occurring subsequent to separation of the *M. truncatula* and Arabidopsis lineages: (a) extensive segmental duplication, accompanied by (b) chromosomal rearrangement and (c) extensive erosion of the structure of duplicated regions. On the basis of a comparison of homologous segmental duplications within each genome, we infer that the nature and extent of within-genome degradation of microsynteny are largely similar between these two species. Divergence between these two genomes was also evident in the form of genes in *M. truncatula* that lack close homologs in Arabidopsis. Interestingly, some of these genes appear to be expressed specifically during symbiotic nitrogen fixation in *M. truncatula*, a process that is characteristic of legumes, and thus may contribute to legume-specific phenotypes. On the basis of these results, it is evident that Arabidopsis cannot serve as a specific model for the structure of legume genomes. Instead, we anticipate that sequencing efforts on legume genomes, including *M. truncatula* and *L. japonicus* for which genome sequencing projects are currently under way, will provide the basis for a detailed view of legume genome structure.

MATERIALS AND METHODS

Sequencing of BAC clones (Nam et al., 1999) was performed by a shotgun approach. Completion of individual BAC clones involved paired-end sequencing from subclones to obtain a 7- to 8-fold average depth of coverage. For BAC survey sequencing, 96 randomly selected subclones were sequenced. The sequences were assembled with PHRAP software package (Gordon et al., 1998; Ewing et al., 1998). The completed BAC sequences were analyzed for gene prediction using GENSCAN (Burge and Karlin, 1997) and GENEMARK.HMM (Lukashin and Borodovsky, 1998) with Arabidopsis settings.

A *Medicago truncatula* genetic map (H.-K. Choi and D.R. Cook, unpublished data; http://www.medicago.org) composed primarily of cleaved amplified polymorphic sequences markers was used to evaluate the level of global synteny between the *M. truncatula* and Arabidopsis genomes. All template sequences of mapped markers (ESTs or BAC end sequences) were first analyzed against the MtGI (http://www.tigr.org/tdb/mtgi) to obtain TCs for individual ESTs or to correlate BAC end sequences with expressed genes. Assignment of a genomic sequence to a TC or an EST was allowed when two sequences overlapped for at least 40 bp.

BLASTX searches (Altschul et al., 1997) were performed against the Arabidopsis protein database (http://www.Arabidopsis.org). Where possible, *M. truncatula* TCs were used as query sequences. For the annotated BAC sequences, the predicted genes were used as query sequences for BLAST searches.

We used several criteria to filter the BLAST results. Significant matches were claimed only when (a) the BLAST expected value was less than e^{-30}, (b) the amino acid sequence alignment showed at least 50% identity, and (c) the BLAST output suggested low copy number in the Arabidopsis genome (typically less than three; see supplemental data). In cases of short query sequences (<500 bp), hits with an e value $< e^{-30}$ and an identity of >60% were considered to be significant. The physical positions of Arabidopsis genes on chromosomes were obtained by querying Map Viewer (http://www.Arabidopsis.org) with the Arabidopsis BAC clone IDs.

Putative orthologs between *M. truncatula* and Arabidopsis were obtained from the TIGR MtGI database and review of the corresponding orthologous gene alignments (Lee et al., 2002). Sequence alignments and phylogenetic analysis were performed using the ClustalX (Thompson et al., 1997). Phylogenetic trees were constructed using the neighbor-joining method as implemented in ClustalX with 1,000 bootstrap sampling steps. The nonsynonymous nucleotide substitution rates (K_a) were calculated using MEGA v2.1, using the method of Kumar et al. (2001).

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