[1]Bioenergy, Genomics, and Accelerated Domestication: A U.S. Example

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The U.S. consumes roughly 26% of the world's energy; yet we represent about 6% of the world's population. In 2005, the U.S. energy supply (105 quadrillion British Thermal Units [BTUs]) was derived from four major sources: coal (23.05 quadrillion BTUs), natural gas (18.76 quadrillion BTUs), crude oil (10.94 quadrillion BTUs) and petroleum (28.87 quadrillion BTUs), with only 6.06 quadrillion BTUs coming from renewable sources including wind, solar and biomass (http://www.eia.doe.gov/). Of this, 69.17 quadrillion BTUs comes from domestic production and the rest comes from imports. Energy consumption fell into four main categories: residential (21.87 quadrillion BTUs), commercial (17.97 quadrillion BTUs), industrial (31.98 quadrillion BTUs) and transportation (28.06 quadrillion BTUs). The transportation sector is unique in that 98% of its consumption comes from petroleum and crude oil, making it most dependent upon foreign imports. Domestic production has declined over the past 20 years and is expected to continue to decline. Annual consumption is growing and imported oil is meeting this demand. Continuing with business as usual has economic, environmental and national security consequences.

One predicted environmental consequence is global climate change due to elevated CO_2 and other greenhouse gases in the atmosphere. CO_2 levels exceeded 370 parts per million for the first time in modern history in 2000 and have continued to climb since then (http://cdiac.ornl.gov/ftp/trends/co2/maunaloa.co2). The U.S. is currently the leading producer of CO_2 at 5.9 billion metric tons per year. The U.S. produces more CO_2 than all of Central and Southern America and Africa combined. As the standards of living increase in the developing world other nations will equal or exceed the annual U.S. CO_2 output, with China expected to surpass the U.S. in 2008.

In the U.S., the single energy sector most affected by limited supplies and higher cost is the transportation sector. There are alternatives to the current petroleum based supply, including coal to liquid, gas to liquid, heavy oil production, deep water production and hydrogen. Among these there are various sources of energy options for meeting the demand for environmentally friendly, affordable new sources of liquid transportation fuels. The question becomes how do we reduce our need for imported sources of energy? Maintain our standard of living? Reduce our carbon emissions? Preserve our energy security? Maintain our reliance on affordable transportation fuels? Closed–loop domestic production of lignocellulosic biofuels provides the most viable solution. The question then becomes how do we create closed–loop domestic production of lignocellulosic biofuels.

Only 6% of the current U.S. energy supply comes from renewable sources and of that only 0.036% is in the form of liquid transportation fuels. Solar (0.006% of the total), wind (0.0012%),

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hydroelectric (0.03%) and direct combustion of biomass (0.02%) make up the remainder. Of the 548,000 vehicles in the U.S. in 2005, the U.S. has increased its flexible fuels vehicles over the past 5 years to approximately 146,000, yet ethanol consumption remains minimal (http://www.eia.doe.gov/) though increasing annually. The majority of the ethanol sold in the U.S. comes from corn grain, though it is estimated the 1 billion tons of biomass could be made available annually for conversion to liquid transportation fuels (http://www.osti.gov/bridge). Of this, closed–loop domestic production of lignocellulosic biofuels represents one third of the total billion tons or 8.0 quadrillion BTUs or 20% of the U.S. transportation sector demand by 2020. Improvements in both the production of the biomass feedstock and in the conversation process will have to be achieved to meet this target. Genomics has a role to play in both the production and conversion of biomass into liquid transportation fuels (U.S. Department of Energy, 2006).

The Role of Genomics. The U.S. Department of Energy, Office of Science through its Joint Genome Institute (JGI) has focused on providing near-term deployment of genome-based tools to hasten the development of new sources of energy and chemical feedstocks. The process of creating a lignocellulosic-based biofuel begins with the fixation of energy from the sun in the form of glucose which is subsequently converted to cell wall polymers (*i.e.*, cellulose, hemicellulose and lignin) and other plant metabolites. Once harvested, these cell wall polymers (*i.e.*, lignocellulosic feedstocks) are then broken down into sugars (*i.e.*, saccharification) and fermented into alcohols (*i.e.*, fermentation) by enzymes and microbes (http://www1.eere.energy.gov/biomass/).

Dedicated biomass feedstocks (as opposed to crop wastes and residuals) come in a variety of forms. The JGI is sequencing, assembling and annotating several candidate biomass species along with several model genomes. For the monocots, draft genomes from *Sorghum*, *Brachypodium*, *Maize* and *Setaria* are or will be available in the next few years. *Sorghum* and *Maize* are bona fide biomass species, whereas *Brachypodium* and *Setaria* serve as model genomes for the larger *Triticale* and *Panicum* and *Miscanthus*, respectively. For the dicots, draft genomes from *Populus*, *Glycine*, *Prunus*, and *Eucalyptus* are or will be available in the next few years. *Populus* and *Eucalyptus* are bona fide biomass species, *Glycine* is a current biodiesel crop and *Prunus* is a model genome for all Eudicots.

Many of the enzyme systems needed for saccharification currently exist in the guts of biomass dependent organisms such as termites and ruminants. The JGI is currently sequencing the metagenomes of the Tammar wallaby forestomach, poplar biomass degraders, elephantgrass decomposers, and Asian long-horned beetle gut, as well as wood degrading organisms such as *Phanerochaete chrysosporium*, *Clostridium thermocellum*, *Saccharophagus degradans* and *Acidothermus cellulolyticus*. Discovery of genes from these organisms and communities will be sources of novel enzymes for the breakdown of plant cell walls (Graber *et al*. 2004). Similarly, a suite of microorganisms have been selected and are being sequenced to support the fermentation process. These species include *Thermoanaerobacter*, *Ethanolicus*, *Pichia stipitis* and others (Lammers *et al*. 2004, Martin *et al*. 2004). These organisms are known to convert five and six carbon sugars into alcohols. Increasing the capacity for rapid fermentation through a genome-illuminated understanding of enzyme function and regulation will enhance the productivity and sustainability of lignocellulosic derived ethanol.

Modern Plant Genomics. The goal of modern plant genomics (including proteomics -- an organism- or tissue-wide analysis of protein regulation, expression, structure, post-translational modification, interactions, and function and metabolomics -- an analysis of the total small molecule complement within an organism or tissue, etc.) is to understand how plants do what

they do. The tools of biotechnology are currently being employed to discover the function of every gene in several diverse plant species, including *Arabidopsis*, rice and *Populus*, among others. Eventually, we will know: 1) the function of each gene, 2) the cells in which each gene functions and when, 3) the relationship each gene has with all other genes and 4) the consequences of mutated gene function. This type of comprehensive functional information about plant genomes is essential to developing efficient and effective strategies for plant improvement. Traditionally, these needs have been perceived as food, feed and fiber, but our dependence upon fossil fuels and the concomitant impacts on global carbon cycle now place bioenergy among the benefits we may derive from plants.

The Arabidopsis and rice genomes. Both the Arabidopsis and rice genomes have been sequenced and annotated (*i.e.*, gene structure and position have been predicted) as part of large international collaborations (Arabidopsis Genome Initiative 2000, Goff *et al*. 2002). From these and all other plant-based genomics datasets, there are now tens of thousands of known and putative genes publicly available. Many of these genes are relevant to accelerated domestication efforts in *Populus* and *Panicum*. This library of genes is expected to grow exponentially over the next several years. In fact, the goals and objectives of the current 5-year Arabidopsis Science Plan are to completely understand the biology of Arabidopsis, to develop an expanded genetic toolkit which would enable scientists to conduct functional genomics research, and to identify the whole-system of gene function, including genome-wide analyses of gene expression, the plant proteome, metabolite dynamics, molecular interactions and comparative genomics [http://www.nsf.gov/pubs/2002/bio0202/2010report.pdf]. Access to this and other similar databases will provide the scientific foundation for accelerated domestication in *Populus* and *Panicum*.

The *Populus* Genome Project. The U.S. Department of Energy, Office of Biological and Environmental Research has created a 7.5x draft sequence of the *Populus* genome (Tuskan *et al*. 2006). A female *Populus trichocarpa* clone, 'Nisqually-1', was used as the representative template for all *Populus* genotypes. The genus *Populus* is especially well suited to serve as the model genome for trees. *Populus* has: 1) small genome size -- the haploid genome size is only ca. 480 Mbp (*i.e.*, 10^6 base pairs), similar to rice, only 4 times larger than *Arabidopsis*, and 40 times smaller than pine, 2) rapid juvenile growth -- allowing meaningful measures of important traits to be documented within a few years after establishment of genetic trials, 3) ease of clonal propagation -- allowing manipulations to be evaluated across common genetic backgrounds, destructive sampling (*e.g*., wood quality assessment) without loss of the genotype, replication of experiments across time and space, and genetic stocks to be archived in clonal nurseries to be shared with researchers around the world, 4) high-throughput transformation (*i.e.*, genetic engineering) and regeneration -- transgenic trees can be produced allowing detection and characterization of gene function (*Populus* is unique among tree genera in this regard), 5) extensive genetic maps -- including the initial identification of quantitative trait loci (QTL) (*i.e.*, DNA markers associated with a wide variety of traits), and 6) existing collaborative network of scientists around the world who have used poplars as a model organism to study tree morphology, physiology, biochemistry, ecology, genetics, and molecular biology (http://www.ornl.gov/ipgc/). *Populus* is only the second dicotyledonous plant to have its complete genome sequenced, *Arabidopsis* being the first. The *Populus* database is a valuable resource for those studying basic plant growth and development as comparative tools for studying gene function. The 7.5x draft of the *Populus* genome along with the complete assembled and annotated genome is currently available.

Gene Regulation Prospects. One avenue for the application of genomics to the development of biomass energy crops is through targeted gene expression. Here candidate genes would be identified within genomic libraries from current agronomic species. The sequence information would be used to locate the homologous domestication genes in either *Populus* or *Panicum*. Once identified, gene insertion constructs would be created to control the expression of such genes. Two types of gene transformation constructs could be used for each candidate domestication gene/homolog: an ectopically expressed, dominant, up-regulated, gain-of-function transgene under the control of a constitutive promoter and a panhandle construct designed to down-regulate candidate gene expression via RNA interference (Waterhouse *et al*. 1998, Chuang and Meyerowitz 2000) to generate a dominant loss-of-function mutant (Filichkin *et al*. 2007). To make the up-regulated construct, each candidate gene would be identified in the genomic library, in the case of *Populus*, or in the case of *Panicum* amplified from its bacterial artificial chromosome (BAC) library by polymerase chain reaction (PCR) using a proofreading polymerase.

The simplest, most robust, and most effective suppression of endogenous genes is currently achieved with a panhandle transgene under the control of a strong transcriptional promoter. The panhandle reliably produces a dsRNA molecule that triggers the degradation of the cognate mRNA through what is presumed to be a targeted RNA endonuclease. Chuang and Meyerowitz (2000) showed that four panhandle constructs were 87-99% (mean = 93%) effective at producing the desired mutant phenotypes in transgenic *Arabidopsis*. Further, they demonstrated that independently-derived lines of transgenic *Arabidopsis* containing panhandle constructs produced an allelic series of varying strength, suggesting that panhandles can provide a complete spectrum of gene suppression from no suppression to complete suppression. Such a 'graded series' of phenotypes is highly desirable, since it permits selection for optimal intermediate levels of gene expression somewhere between 'ON' and 'OFF.' Panhandles would be designed to avoid highly conserved motifs within the candidate gene to prevent unwanted suppression of entire gene families (Chuang and Meyerowitz 2000, Filichkin *et al*. 2007).

Plant domestication. Plant domestication is the conversion of a wild plant, whose morphology and physiology are directed towards survival and reproduction in nature by the process of natural selection, into a cultivated plant whose morphology and physiology have been drastically altered by artificial selection as a means of increasing the yield of useful products. Under modern agricultural systems, domesticated plants are highly productive. Domestication through traditional means of plant breeding and agronomic modifications has resulted in advances in overall growth, pest resistance and adaptation (Duvick 1996). Increasing the human and financial resources available to a traditional domestication approach would hasten future advances in dedicated energy crops, but the perennial nature and large plant sizes inherent to most biomass crops constrain progress as a result of delayed reproduction and inaccessibility to flowering tissues. Leveraging modern molecular genetics techniques will allow us to accelerate the domestication process in biomass species such as *Populus* (Wullschleger *et al*. 2002) and *Panicum* (McLaughlin and Kszos 2005). Accelerating the domestication process would increase average plant and/or stand productivity, improve overall feedstock uniformity and reduce the cost of supplying conversion facilities with biomass feedstocks.

Maize Domestication. Teosinte is widely believed to be the progenitor of modern corn (Paterson *et al*. 1995, Doebley 2004). From this non-domesticated, low-grain yielding, shrubby-statured perennial grass, Native American farmers roughly 5000 years ago were able to produce a high-yielding agronomic grain -- maize. By the time European explorers discovered the Americas, maize was a staple food source for much of Central and North America. Indian corn had no

lateral branching, a non-dehiscing seed head (*i.e.*, seed remain on the plant after maturing) and a soft seed coat. These traits were fixed early in the domestication process and involved a relatively small number of genes (Doebley 2004). Similar genetic changes have been documented in other domesticated crops as well. After these early domestication steps took place, further improvements through the application of modern breeding techniques resulted in additional gains in yield, product quality, pest resistance, etc. (Wright *et al.* 2005). Still, the most profound advancement in corn yields occurred early in the domestication process and involved a relatively small number of genes. Today modern corn is planted at a density of 63,000 plants per hectare and has achieved yields as high as 440[+] bushels per hectare (Duvick 1996).

***Populus* Domestication.** For *Populus*, the modern domestication process began only recently. The cultivation and propagation however began centuries ago. The Romans were probably the first people to utilize *Populus* in cultivated settings, planting poplar trees in urban areas. The Romans are also believed to have coined the generic moniker, *Populus, i.e.*, the People's Tree. The next major event in the domestication of *Populus* was the use of unrooted poplar by the Napoleonic armies as a means of identifying snow-covered roadways in the winter months in northern Europe. In the spring, army engineers would drive short dormant poplar sticks into the ditches lining the roads, by winter tall young trees lined the roads, allowing the mobile army to avoid the soft soil in adjacent fields. Active poplar breeding did not begin until the mid-twentieth century, when plant breeders in Europe and North America were selecting and crossing individual parents with the objectives of increasing adaptability, pest resistance and overall growth (Bradshaw *et al.* 2000). These crossing efforts were successful and led to improved hybrid progeny that were then vegetatively propagated.

Fully Domesticated Woody Crops. Many "domestication" genes have been identified from respective agronomic crops (Wright *et al.* 2005, Hufford *et al.* 2007). Leveraging this information in a biomass energy context, along with the enormous genomics database from *Populus* (Tuskan *et al.* 2006), we can accelerate the domestication process for energy crops. For example, genes for height growth, response to competition, branching, stem thickness and cell wall chemistry are now available to test the hypothesis that *Populus* can be domesticated more rapidly using modern molecular genetics approaches. *Populus* genes will be identified based on sequence homology to "domestication" genes from other crop species. These native genes will be up- or down-regulated (*i.e.*, over or under expressed) in transformed plants. Thus, domesticated energy crops would contain no foreign DNA.

Domestication Case Study -- *Populus*. A fully domesticated *Populus* clone would accumulate greater carbon allocation in the stem, through the development of a less extensive root system, reduced height and minimal proleptic (*i.e.*, perennial branch formation and growth) branching (Wullschleger *et al.* 2005). By regulating the distribution of carbon within a plant itself, it is possible to increase the harvest index (*i.e.*, the amount of harvested biomass that can be used in an energy application) without having to increase the net productivity of the plant. A fully domesticated poplar will be relatively short but with a large stem diameter, generating lower amounts of low quality wood (*i.e.*, reaction wood), higher harvest index, and improved harvesting/handling efficiencies. Because of the steep water potential gradient that occurs with tall trees as water is transported from the base to top of the tree, reduced height should result in higher water potential in stems and leaves, enabling photosynthesis to continue at maximal rates longer during dry, sunny days when vapor pressure deficits cause stomatal closure. A domesticated poplar will have reduced numbers and sizes of branches, again favorably impacting xylem conductivity and internal water status. A domesticated *Populus* clone would also display minimal or no response to light competition and would have an optimized photoperiod response

for each growth environment. Such a plant could be established at high densities, allowing greater per unit area productivity. These plants would also display optimal leaf area indices over the course of the full growing season, releasing vegetative buds as early as possible in the spring and holding leaves as long as possible in the fall. A domesticated *Populus* clone would also express stable pest resistance over the course of a growing season, a full rotation and/or over successive rotations. Finally, a fully domesticated *Populus* clone would not produce flowers. This would again lead to greater allocation of biomass to the stem but more importantly, it would minimize the chances of gene flow out of the plantation.

Because the great majority of carbon contained in biomass is present in cell walls in the forms of lignin, cellulose and hemicellulose, the cell walls of a fully domesticated *Populus* or *Panicum* plant would be modified to produce optimal feedstocks for energy conversion (Ragauskas *et al*. 2006, Sanderson *et al*. 2006, respectively). Changes in cell wall chemistry and/or anatomy will be customized to match the various conversion technologies (see Dinus *et al*. 2001, Vogel and Jung 2001 for further discussions). Through previous efforts in Arabidopsis, rice, etc., all major enzymatic steps in lignin biosynthesis have been identified (Chen and Dixon 2007), as have many of the genes involved in cellulose and hemicellulose biosynthesis (Molhoj *et al*. 2002). Still, the biochemical pathways that produce cell wall polymers are complex, requiring many different enzymes and the genes that encode them (Somerville 2006). It is estimated that approximately 10-15% of genes in plant genomes are directly involved in cell wall biosynthesis, assembly, maintenance and/or modification. Therefore, an early focus for carbon management efforts should be the acquisition of a comprehensive understanding of how plant cell wall polymers are synthesized, how cell wall polymers are assembled into a functional architecture, how and why functional properties differ among different cell types, and how cell walls are degraded in the environment. This understanding would then be applied to domesticate *Populus* via cisgenesis to obtain the desired feedstock composition.

For biochemical conversion processes it may be advantageous to increase the polysaccharide content at the expense of lignin up to a certain point. Since product yield is a critical economic factor for such processes and only the polysaccharide components are converted into product, this strategy makes sense. Conversely, on a weight basis the heat of combustion of lignin is greater than that of polysaccharide. Therefore, for thermochemical conversion processes it makes more sense to tune the balance of cell wall polymers to increased lignin content (Dinus *et al*. 2001). Finally, we expect that domesticated poplars will no longer survive in the wild. Cultivation by humans will provide an environment free from light, water, nutrient or competitive stresses. Much the same as domesticated crop plants; outside this environment, domesticated poplars will be maladapted for survival.

A change in the above traits in *Populus* would have a positive impact on the cost per ton of biomass, thus favorably impacting the amount of land economically available to this dedicated energy crop and ultimately increasing the amount of fossil fuels displaced by biomass feedstocks. Based on the above traits, production costs for domesticated closed-loop energy cropping supply system would be reduced. In a conventional cropping system at a 2.5 m x 2.5 m spacing on a 8-year rotation, the final yield could reach ca. 21 Mg ha^{-1} yr^{-1} (1 Mg = 1 metric ton) from an average tree of 20 m in height and 20 cm in diameter, and would cost approximately \$57 Mg^{-1} to produce, harvest, and process in the field. In the anticipated domesticated closed-loop cropping system, spacing would be reduced to 1.2 m x 2.5 m per tree; rotation age would be reduced to 5 years; yields would be increased to 28 Mg ha^{-1} yr^{-1} from an average tree of 6 m in height and 38 cm in diameter. The actual productivity per tree in terms of carbon fixed per tree would not change, the distribution of the fixed carbon would change and the number of trees per unit area

would increase. The costs associated with harvesting and handling would be reduced to $9 Mg^{-1} and the total cost of growing, harvesting and processing would become $23 Mg^{-1}. These modifications would cause closed-loop energy-cropping systems to be more competitive with both conventional agricultural crops and traditional fossil fuels. As a result, the cost of displacing fossil fuels with biomass-based fuels will be reduced and the potential supply of biomass energy feedstock per unit of land will be increased, thus positively impacting greenhouse gases mitigation (Tuskan & Walsh 2001).

Finally, we have been able to leverage the information in the *Populus* genome and use it in a comparative way in conjunction with the *Arabidopsis* genome database to begin the accelerated domestication process in *Populus*. The AUX/IAA and ARF genes are transcription factor genes that regulate gene expression by initiating or preventing gene transcription. In *Populus* there are 40 members of the ARF gene family whereas Arabidopsis has only 23 members (Kalluri *et al.* 2007). By systematically generating dominant loss-of-function mutants for each gene in each gene family we were able to identify several mutants that have relevance to accelerated domestication in *Populus*. For PoptrIAA7.1 and PoptrIAA7.2, orthologs with AtIAA7 and AtIAA14, RNAi-mediated down-regulation results in severe dwarf phenotype in *Populus* with exaggerated lateral shoot growth. AtIAA7 loss-of-function mutant have phenotype similar to wild-type but has slightly longer hypocotyl and altered shoot gravitropism and AtIAA14 loss of function mutants appears normal. RNAi-mediated down-regulation of PoptrIAA16.31, an ortholog of AtIAA16, resulted in enhanced radial growth in *Populus*; AtIAA16 loss-of-function mutants expressed no visible phenotype. These two mutants appear to control stem elongation and diameter growth consistent with the ideotype described above for a fully domesticated *Populus* clone.

Conclusions. Energy consumption is expected to rise in the U.S. and globally. Emissions of CO_2 will likely also increase with accompanying warming of the planet. Closed-loop dedicated bioenergy crops could offset CO_2 emission by displacing petroleum-based transportation fuels and contributory contributions to carbon sequestration via below ground biomass. Application of genomics-based science can accelerate the domestication of many candidate bioenergy crops through identification and modification of genes controlling key characteristics (*e.g.*, cell wall, carbon allocation, adaptive and physiologic traits). The U.S. Department of Energy and the Joint Genome Institute are committed to facilitating the achievement of these deliverables.

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