Analysis of the U.S. Department of Agriculture's Regulation of Genetically Engineered Crops and Reproductive Biology of *Carex pensylvanica* (Lam.)

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Dedication

This dissertation is dedicated to my daughter, Sophia Keller. I love you and want you to see that all things are possible with faith and hard work.

Abstract

This dissertation is divided into two parts. Chapters 1 through 3 are interdisciplinary and focus on legal and scientific perspectives regarding the regulation of genetically engineered crops. Chapters 4 and 5 evaluated the environmental factors that control flowering in Pennsylvania sedge (*Carex pensylvanica* Lam.).

The commercial potential of genetically engineered (GE) crops has not been fully realized in the United States due to environmental litigation that dramatically affected the pace of GE crop development, testing and deregulation. The United States Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) regulates GE crops. However, litigation initiated by nongovernmental organizations exposed APHIS's vulnerability to lawsuits under the National Environmental Policy Act. APHIS failed to differentiate between traditional GE crops whose risks are well characterized and novel GE crops such as glyphosate-tolerant sugarbeet that may raise unique environmental risks and societal issues based on their distinctive biology. Consequently, we concluded in chapters 1 and 2 that APHIS did not adequately evaluate the legally defined environmental risks of these novel crops, and thus left itself open to litigation.

In 2011, APHIS announced that it lacked the authority to regulate Scotts Miracle-Gro Company's (Scotts) GE Kentucky bluegrass variety. As a consequence of this determination, Scotts is able to field test their new grass without government oversight. Furthermore, Scotts may distribute and sell this product to U.S. consumers without going through APHIS's costly and time-consuming deregulation and environmental assessment process. This was an unexpected development and provides a blueprint for other

biotechnologies to circumvent the regulation and environmental assessment of GE plants. In Chapter 3, we summarized relevant regulations and statutes, described how Scotts was able to avoid regulation, and discussed other emerging technologies that are eluding regulation through piecemeal regulatory decisions. We concluded that the creation of a non-plant pest loophole is a consequence of APHIS's obsolete plant pest regulations, political gridlock between USDA's core constituencies, and a decade's worth of environmental litigation. A comprehensive overhaul of APHIS's regulatory system is needed to assess the environmental risk of crop traits independent of their method of creation.

Pennsylvania sedge (*Carex pensylvanica* Lam.) is an upland forest sedge with restoration and horticultural potential as a low maintenance groundcover for dry shade. For large plantings, achenes are preferred, but Pennsylvania sedge typically produces few achenes in its native habitat. As a first step in improving achene production, Chapter 4 evaluated the effect of vernalization and photoperiod on floral initiation and development. We concluded that Pennsylvania sedge is an obligate short day plant that does not require vernalization for flowering. Plants flowered when exposed to daylengths of 6 to 12 hours. Flowering was completely inhibited with 14-hour photoperiods. Pennsylvania sedge was florally determined after 4 weeks of 8-hour photoperiods. However, the largest number of normal inflorescences were produced with 6 to 10 weeks of 8-hour photoperiods. Inflorescence quantity varied by genotype.

Pennsylvania sedge is a monoecious, dichogamous forest sedge with temporal and spatial separation between male and female flowers. In its native habitat, Pennsylvania

sedge is protogynous; the stigmas emerge and become receptive prior to anthesis. Chapter 5 examined the environmental factors that control floral gender sequence and inflorescence culm heights in a series of four experiments. Plants were first subjected to three short day inductive photoperiods (8-, 10-, and 12-h) to evaluate how this impacted inflorescence height and dichogamy. The second experiment analyzed how different light spectra and temperature fluctuations affect floral stem height and dichogamy. The third experiment sought to determine the approximate date of floral bud initiation in the northern United States by digging field-grown plants at 2-week intervals during the fall and transferring them to a greenhouse under non-inductive photoperiods. The third and fourth experiments evaluated the effect of a chilling treatment on plants that were already induced. Photoperiod, light spectra, and temperature fluctuations failed to produce protogynous flowering. Plants were found to be determined and florally initiated in the fall in the northern United States. A post-floral induction chilling treatment (winter) was necessary to produce protogynous flowering and normal inflorescence culm elongation.

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Chapter 1

Sweet and Sour: A Scientific and Legal Look at Herbicide-Tolerant Sugar Beet

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American sugar beet farmers produce half of our domestic refined sugar valued at over $3 billion per year (APHIS, 2010). The U.S. District Court for the Northern District of California (CA District Court) will soon determine whether U.S. farmers can continue to grow genetically modified (GM) sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima*) in the case of *Center for Food Safety v. Vilsack* (*CFS v. Vilsack*). The CA District Court is grappling with imposing an ambiguous and evolving legal standard on a scientific question of gene flow risks. It will analyze the likelihood of cross-pollination between genetically modified sugar beet and organic Swiss chard or table beet seed crops and determine whether this constitutes irreparable harm. The U.S. Supreme Court is also interested in biotechnology law this term and may overturn precedent that would normally guide the CA District Court. Here, we review the legal and scientific background of this issue, and argue that the CA District Court should consider the scientific as well as the legal factors of this case and impose sensible geographic restrictions that will preserve coexistence among production methods.

Legal History

In 2005, after preparing a concise environmental assessment (EA) under the National Environmental Policy Act (NEPA), the USDA Animal and Plant Health Inspection Service (APHIS) unconditionally approved genetically modified glyphosate-tolerant sugar beet and found no significant environmental impact (USDA/APHIS, 2005). U.S. farmers embraced Roundup Ready sugar beet (RR sugar beet) for its superior weed control and planted 95% of U.S. acreage in RR sugar beet by 2009 (APHIS, 2010). Plaintiffs Center for Food Safety, Organic Seed Alliance, Sierra Club, and High Mowing

Organic Seeds brought suit against APHIS (naming the Secretary of the USDA, Tom Vilsack) challenging its decision to deregulate RR sugar beet and asserting that APHIS violated NEPA. The CA District Court ruled in the first phase of *CFS v. Vilsack* in 2009 that APHIS violated NEPA because it should have prepared an environmental impact statement (EIS) assessing gene flow to related cultivated species (Table 1 provides a list of this and other court cases mentioned in this article). Judge White based his decision on the socio-economic risk that RR sugar beet seed crops could cross-pollinate with conventional or organic Swiss chard or table beet seed crops in the Willamette Valley in Oregon (Figure 1). In particular, he judged that APHIS failed to take into account that transgenic pollen flow would deprive conventional or organic seed farmers of their choice not to grow GM crops and consumers of their choice not to consume GM foods. While the CA District Court stated only one basis for finding a NEPA violation, Judge White also articulated in detail the potential for introgression of the glyphosate resistance gene into wild and hybrid weed beet species in the Imperial Valley, CA.

In the second phase of this two-part decision, the CA District Court will hear oral arguments on July 9, 2010 to determine whether a permanent injunction should issue to halt all or a portion of RR sugar beet seed and root production pending preparation of the EIS. Monsanto Company, Betaseed, Inc., Syngenta Seeds, Inc., SES Vanderhave USA, Inc. and their allies in the sugar processing industry intervened in this second phase in defense of RR sugar beet production. The outcome of the injunction motion will have nationwide ramifications.

The CA District Court and the Ninth Circuit Court of Appeals (Ninth Circuit) are the only American courts to have previously ruled on APHIS's deregulation of other

genetically modified crops. The CA District Court in *Geertson Seed Farms v. Johanns* (*Geertson v. Johanns*), issued an injunction in 2007 prohibiting the continued planting of glyphosate-tolerant alfalfa (RR alfalfa) pending preparation of an EIS, and the Ninth Circuit affirmed this decision in 2009. Farmers that planted RR alfalfa prior to March 30, 2007 may continue to harvest their fields subject to strict stewardship guidelines. Legal developments continue to affect RR alfalfa. APHIS released a draft EIS supporting the deregulation of RR alfalfa in November, 2009 and is still processing public comments. In the interim, Monsanto appealed the Ninth Circuit's decision to the Supreme Court (*Monsanto v. Geertson*) and the decision is pending. Therefore, the future of RR sugar beet and RR alfalfa is uncertain.

Environmental Risk and Benefit of RR Sugar Beet Cultivation

Minnesota, Idaho, North Dakota, and Michigan together produced 82%, and California 3%, of the nation's sugar beet crop in 2009 (Figure 1; http://usda.mannlib.cornell.edu/usda/current/CropProdSu/CropProdSu-01-12-2010.pdf).Sugar beet plants are biennial. In its first year, a sugar beet produces a rosette of leaves and a large storage root containing sucrose. During its second year, the sugar beet will flower if exposed to vernalizing temperatures and lengthening photoperiod (Milford, 2006). Excluding California, the U.S. crop is planted in the spring and the roots are harvested in late fall. During production, < 0.01% of plants flower and the plants do not survive winter (APHIS, 2010). Farmers growing RR sugar beet contractually agree to remove any flowering plants in the year of production. Sugar beet plants are more likely to flower in California than in Midwestern and Pacific northwestern root production

states (Monsanto, 2003). California farmers plant sugar beet as a winter crop and it may receive sufficient vernalization to flower. However, California farmers routinely cut the 1.5 m flowering stalk to prevent yield loss because it competes with the root for sucrose allocations and interferes with harvest. In contrast to root production, the vast majority of seed production of RR sugar beet takes place on 3,000 to 5,000 acres in the Willamette Valley, OR. The seed crop is planted in the fall, vernalized during the moderate winter and allowed to flower the following year. Sugar beet seed crops share the Willamette Valley with closely related specialty seed crops such as Swiss chard and table beet. Most table beet and Swiss chard seed crops are grown in Washington and California. Therefore gene flow among these three sexually compatible crops is a cause for environmental concern only in the Willamette Valley.

Weed pressure can significantly limit sugar beet root production (USDA, 2005). In fields of conventional sugar beet, weeds typically are partially controlled with micro rate applications of several herbicides (May and Wilson, 2006). Satisfactory weed control often is elusive, and farmers typically supplement their herbicide use with cultivations and manual weed removal. The 2008 survey of Minnesota and North Dakota sugar beet weed control practices showed that RR sugar beet with glyphosate resulted in the highest weed control ratings in the history of the survey and also nearly eliminated mechanical and manual weeding (http://www.sbreb.org/research/weed/weed08/surveyweedcontrol.pdf). The survey also showed that post-emergence herbicide use was at its lowest level since 1991. In addition to limiting herbicide use, RR sugar beet cultivation may reduce energy use and ecotoxicity (Bennett et al., 2004). The use of RR sugar beet may also lead to the adoption

of more sustainable cultivation practices including mulch systems, winter cover crops and strip tilling (Petersen and Röver, 2005; May, 2003; Overstreet, 2009). Strip tillage is now more feasible because farmers do not need to cultivate to control weeds in RR sugar beet fields.

Using glyphosate-tolerant (GT) crops increases the probability for development of resistant weeds (Duke and Powles, 2009). If farmers were to plant two or three successive Roundup Ready® crops (such as sugar beet, corn, and soybeans), this would further increase selection pressure for such weeds. Maintaining diversity in crop rotation (GT and non-GT crops) coupled with the use of herbicides with different modes of action is an important factor in lessening this risk (Duke and Powles, 2009).

Risk of Gene Flow to Cultivated and Weedy Relatives

Sugar beet, Swiss chard (*B. vulgaris* L. ssp. *vulgaris* var. *flavescens)*, table beet (*B. vulgaris* L. ssp. *vulgaris* var. *vulgaris*), sea beet (*B. vulgaris* L. ssp. *maritima* (L.) Arcang.) and *B. macrocarpa* Guss. are inter-fertile (OECD, 2001) and are predominantly wind-pollinated. The presence of wild beet species such as *B. macrocarpa* and *B. vulgaris* ssp. *maritima* in close proximity to RR sugar beet poses concerns over transfer of the glyphosate resistance gene. Given the similarity in herbicide sensitivity, the presence of a glyphosate-resistant weed beet would be problematic to control. Numerous sugar beet pollen flow studies have been published with varied methodology and plant material. Wild beet species and hybrid weed beet are a serious problem in sugar beet production fields in western Europe (Boudry et al., 1993). Hybrid weed beet differs from cultivated sugar beet in that it displays an annual life cycle that does not require

vernalization. It appears to be the result of wild beet species such as *Beta vulgaris* ssp. *maritima* pollinating cultivated male-sterile female sugar beet plants in seed production areas in Europe (Boudry et al., 1993). Gene flow has been documented from GM sugar beet to hybrid weed beet in European fields at distances of 112 m (Darmency et al., 2007) and 200 m (Alibert et al., 2005). Since gene flow between weed beet populations has been documented up to 9.6 km (Fenart et al., 2007), the potential exists for the creation of glyphosate-resistant weed beet (Darmency et al., 2007). Sugar beet pollen was detected 1,097 m from fields (Dark, 1971) and at an altitude of 1,524 m (Meier and Artschwager, 1938). Outcrossing between GM sugar beet and conventional male sterile sugar beet plants occurred at distances up to 1,000 m (Alibert et al., 2005).

Beta vulgaris ssp. *maritima* and *B. macrocarpa* have been introduced into California and *B. macrocarpa* is present in the Imperial Valley of California, an area of sugar beet root production (Figure 1) (http://ucjeps.berkeley.edu/interchange/index.html). There is evidence of limited introgression from sugar beet into *B. macrocarpa* in the Imperial Valley and evidence of hybridization between escaped table beet or Swiss chard and *B. vulgaris* ssp. *maritima* in the San Francisco Bay area, a non-production area for sugar beet (Bartsch and Ellstrand, 1999). Transmission of the glyphosate resistance gene into *B. macrocarpa* is possible if RR sugar beet is planted in the Imperial Valley because winter planted sugar beet is more likely to be vernalized and to flower. Introgression into *B. vulgaris* ssp. *maritima* is less of a concern in other parts of California because its distribution is outside sugar beet production areas. If glyphosate-resistant *B. macrocarpa* becomes a reality and invades sugar beet fields, it would be a significant problem in production. RR sugar beet has not been grown in the Imperial Valley to date, but limited

cultivar selection of RR sugar beet may be the predominant reason for California growers to abstain from its use, rather than concern over the potential transfer of glyphosate resistance to weedy relatives. The sugar beet industry in California is in decline, especially since the closure of seven out of eight sugar beet processing plants since 1992 (http://www.sugarpub.com/event/article/id/53/).

In contrast to California, the rest of the U.S. does not face significant gene flow risks since flowering does not regularly occur in these areas. Furthermore, the distribution of *B. vulgaris* ssp. *maritima* (www.eFloras.org) is limited to California and New Jersey (a non-sugar beet producing state). *Beta macrocarpa* does not occur outside of California (www.plants.usda.gov). It is possible that isolated hybrid weed beet populations exist outside California because sugar beet seed has been imported from European production areas in the past. However, they currently are not a problem and have not been documented. Thus, Midwestern and northwestern production and seed areas do not face the same risk of gene flow as does California or Europe.

Gene flow may occur from RR sugar beet to conventional and organic Swiss chard or table beet seed crops grown in the Willamette Valley, OR. Organic growers contend that cross-pollination may render seed unmarketable due to the zero or low tolerance for transgenic contamination. Furthermore, transgenic contamination may deprive organic consumers of their choice to consume non-genetically modified vegetables. However, production practices are already in place to limit gene flow among adjacent related crops. The Oregon Seed Certification standard isolation distance requires a minimum 2,438 m isolation distance between sugar beet crops and Swiss chard or table beet (http://seedcert.oregonstate.edu/sites/default/files/standards/sugar-beets-standards.pdf) A

seed industry-established organization, Willamette Valley Specialty Seed Association (WVSSA), decided that the Oregon Seed Certification standards were not adequate and now requires an isolation distance of 4,828 m between RR sugar beet and Swiss chard or table beet (http://www.thewvssa.org/documents/WVSSA_Isolation_Guidelines.pdf). Although membership is voluntary, all Willamette Valley commercial specialty seed producers are members of the WVSSA and as such are obligated to comply with the specified isolation distances (Dahmer, 2008). Betaseed exceeds the 4,828 m isolation distance by requiring a 6,437 m isolation distance (Dahmer, 2008), and other companies may also voluntarily follow this isolation distance. The sugar beet seed industry understands the necessity for distance between related crops; therefore, court-mandated isolation distances may be an effective tool for preventing gene flow.

A requirement for isolation distances is based on the assumption that the glyphosate tolerance trait is carried in the pollen. However, the intervention of SESVanderhave USA, Inc., a sugar beet seed producer, in the second phase of *CFS v. Vilsack* highlights technologies that may limit inadvertent cross-pollination between sexually compatible crops. SESVanderhave and other seed companies produce RR sugar beet seed by using conventional male pollinators that do not carry the glyphosate tolerance gene. Instead, the hybrid inherits glyphosate tolerance from the male-sterile female line from which seed is harvested. Thus, the risk of gene flow via pollen to conventional and organic crops is minimized; however, there is the possibility that an occasional female plant is not male sterile and gene flow could occur through seed escape. Recent court documents allege that 78% of the RR sugar beet crop is produced using conventional male pollinators. It is important that the CA District Court assess this method of seed production when

weighing risk and benefit from RR sugar beet production.

The Supreme Court Interprets NEPA

Recent U.S. Supreme Court activity may influence the scope of injunctive relief against RR sugar beet and underscores the unsettled and evolving nature of NEPA law. The 2008 Supreme Court case *Winter v. National Resources Defense Council, Inc.* (*Winter v. NRDC*) clarified the legal standard for issuing injunctions in NEPA cases. This decision halted automatically-imposed injunctions in NEPA violation cases and was openly critical of the Ninth Circuit's approach to injunctions. The Ninth Circuit is the appellate court that reviews the decisions of the Northern District of California.

In *Winter v. NRDC*, Plaintiffs NRDC and Jean Michael Cousteau challenged the U.S. Navy's use of sonar in detecting submarines during training exercises. Plaintiffs alleged that the Navy's use of sonar would harm beaked whales and other marine mammals and that the Navy should have prepared an EIS instead of an EA under NEPA. The lower court entered a preliminary injunction ordering the Navy to shut down its sonar when it detected a marine mammal. The Navy appealed these restrictions. The Ninth Circuit upheld the lower court's injunction in its entirety on the basis that an injunction may be ordered based on evidence showing the "possibility" of irreparable harm. In reversing the Ninth Circuit, the U.S. Supreme Court ruled that an injunction is an extraordinary remedy that cannot be based upon the mere "possibility" of irreparable harm. Instead, a plaintiff requesting an injunction must demonstrate a "likelihood" of suffering irreparable harm. In addition, the lower court must weigh the respective environmental and economic burdens to the parties and consider whether the injunction is

in the public's interest. *Winter v. NRDC* has broad relevance beyond its narrow set of facts, and a ripple effect has swept the country as lower courts have rushed to articulate the correct standard in injunctive proceedings. Dozens of federal cases (Narodick, 2009) have cited the clarified standard for all types of federal injunctions, including the Ninth Circuit in *Geertson v. Johanns* on RR alfalfa.

Geertson v. Johanns might have had great precedential value for *CFS v. Vilsack* because it involved similar legal questions regarding RR alfalfa in the CA District Court. However, RR alfalfa's fate is uncertain due to the U.S. Supreme Court's decision to review the scope of the permanent injunction in *Monsanto v. Geertson* in 2010. The issues raised by Monsanto on appeal are whether the Ninth Circuit erred in: (1) presuming irreparable harm as opposed to requiring plaintiffs to prove a likelihood of irreparable harm; (2) upholding the CA District Court's decision to enter a broad permanent injunction without requiring an evidentiary hearing; and (3) upholding the CA District Court's injunction that was entered prior to *Winter v. NRDC* and thus was based on the possibility as opposed to the likelihood of irreparable harm. The Supreme Court heard oral arguments on April 27, 2010 and a decision is expected soon. Considering that the Supreme Court overturned four Ninth Circuit environmental cases during the last term, one may suspect that the Ninth Circuit's decision in *Geertson v. Johanns* may not be affirmed in its entirety.

It would be valuable if the Supreme Court in *Monsanto v. Geertson* defined "irreparable harm" in the biotechnology context, because the phrase is fraught with ambiguity. What is the likelihood of irreparable harm in *CFS v. Vilsack*? The Ninth Circuit in *Geertson v. Johanns* in 2009 defined irreparable harm as any amount of

transgenic cross-pollination of neighboring organic or conventional crops. The amount and probability of cross-pollination was irrelevant, and there was no tolerance for low levels of cross-pollination. Unfortunately legal precedent has not defined "likelihood of irreparable harm" in scientific terms. All that is known is that the harm must not be speculative or improbable. Irreparable harm in this context might be defined by a likelihood of significant gene flow to wild species. Irreparable harm may also be defined as the likelihood of exceeding a low-level threshold for GMO material in organic crops. The threshold would need to balance a consumer's preference to consume predominantly organic food with the needs of RR sugar beet growers.

Conclusions and Appropriate Injunctive Remedies

The Plaintiffs in *CFS v. Vilsack* have moved for a permanent injunction banning the further planting, cultivation or processing of RR sugar beet or its seed. Our opinion is that such a broad injunction is not justified based upon the distribution and biology of *Beta* species and upon Supreme Court precedent. The CA District Court must determine the likelihood of irreparable harm in assessing gene flow risks and not summarily presume harm solely based on a NEPA violation. Furthermore, the equitable nature of injunctions and the public interest requirement necessitate a balancing act by the CA District Court. The rights of organic farmers and consumers should not be ignored, but neither should the judicial system ignore the economic burden to farmers that have adopted RR sugar beet technology.

The CA District Court should evaluate the likelihood of irreparable harm and issue a tailored permanent injunction permitting the continued production of RR sugar beet

crops subject to two geographic restrictions that greatly lessen the risk to organic and conventional Swiss chard and table beet industries. The first restriction should preserve coexistence between Swiss chard/table beet and RR sugar beet seed industries by mandating scientifically-based isolation distances in the Willamette Valley, OR. Without sufficient isolation distances, the likelihood of irreparable harm or hybridization between adjacent crops is high. However, no study has confirmed cross-pollination from conventional or genetically modified sugar beet at a distance of 6,437 m, the isolation distance voluntarily adopted by the RR sugar beet industry. Furthermore, the majority of the RR sugar beet industry produces seed in which the Roundup Ready gene is inherited through the male-sterile female plant, which further reduces the risk of gene flow through pollen drift. In light of the Supreme Court standard in *Winter v. NRDC,* the likelihood of irreparable harm becomes speculative and would seem no longer significant beyond this isolation distance. APHIS also supports this isolation distance (APHIS, 2010). Therefore, with strong stewardship guidelines for field sanitation once the seed crop has been harvested, risk can be minimized.

The second geographic restriction should address the possibility of gene flow in the Imperial Valley, CA, and any other California counties where sugar beet production coincides with wild beet distribution. To prevent the introgression of the glyphosate resistance gene into wild species, it is necessary to ban the planting of RR sugar beet in southern California. This restriction will pose minimal hardship because California sugar beet farmers have refrained from growing RR sugar beet to date. However, there is the potential for California farmers to adopt Roundup Ready technology while the EIS is prepared. This restriction would prevent this possibility.

With these two geographic restrictions in place, the CA District Court could permit the continued planting of RR sugar beet in root production areas outside California. There is negligible likelihood of irreparable harm in the sugar bowl states of the U.S. (Figure 1). Sugar beet is harvested in the first year and flowering is minimal. This is not like RR alfalfa hay production where the plant routinely flowers in the first year if the hay is not harvested on time. Root production areas are distant from seed production in the Willamette Valley, OR, and wild beet species are limited to California and New Jersey.

For future cases, it is worth considering that APHIS could have prevented this litigation. As the Ninth Circuit stated in *Geertson v. Johanns* in 2009, APHIS has the authority to impose a few well-analyzed geographical restrictions on crops before deregulating them for commercial production. Federal agencies routinely avoid the time and cost of preparing an EIS by recognizing what constitutes the environmental threshold that requires an EIS and implementing mitigation factors that bring an agency project or decision below the threshold (Karkkainen, 2002). APHIS could have recognized that its initial deregulatory decision failed to analyze the impact on the organic Swiss chard and table beet seed industry in Oregon. The CA District Court should consider the scientific as well as the legal factors of this case and impose sensible geographic restrictions that will preserve coexistence between farming industries.

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Table 1. List of Cited Court Cases.

Case Name	Date	Citation	Court
Center for Food Safety v. Vilsack.	**2009**	2009 WL* 3047227	N.D. California
Geertson Seed Farms v. Johanns.	**2007**	2007 WL* 1302981	N.D. California
Geertson Seed Farms v. Johanns.	**2009**	570 F.3d 1130	Ninth Circuit Court of Appeals
Monsanto Co. v. Geertson Seed Farms**.	**2009**	2009 WL 3420495	Supreme Court of the U.S.
Monsanto Co. v. Geertson Seed Farms	**2010**	130 S.Ct. 1133	Supreme Court of the U.S.
Winter v. Natural Resources Defense Council, Inc.	**2008**	129 S.Ct. 365	Supreme Court of the U.S.

Court cases may be found on legal subscription services such as Westlaw or LEXIS.

*denotes Westlaw citation. These cases were not published in either the Federal Reporter or in the Federal Supplement.

**This is not a court decision. This is Monsanto's Petition for Writ of Certiorari.

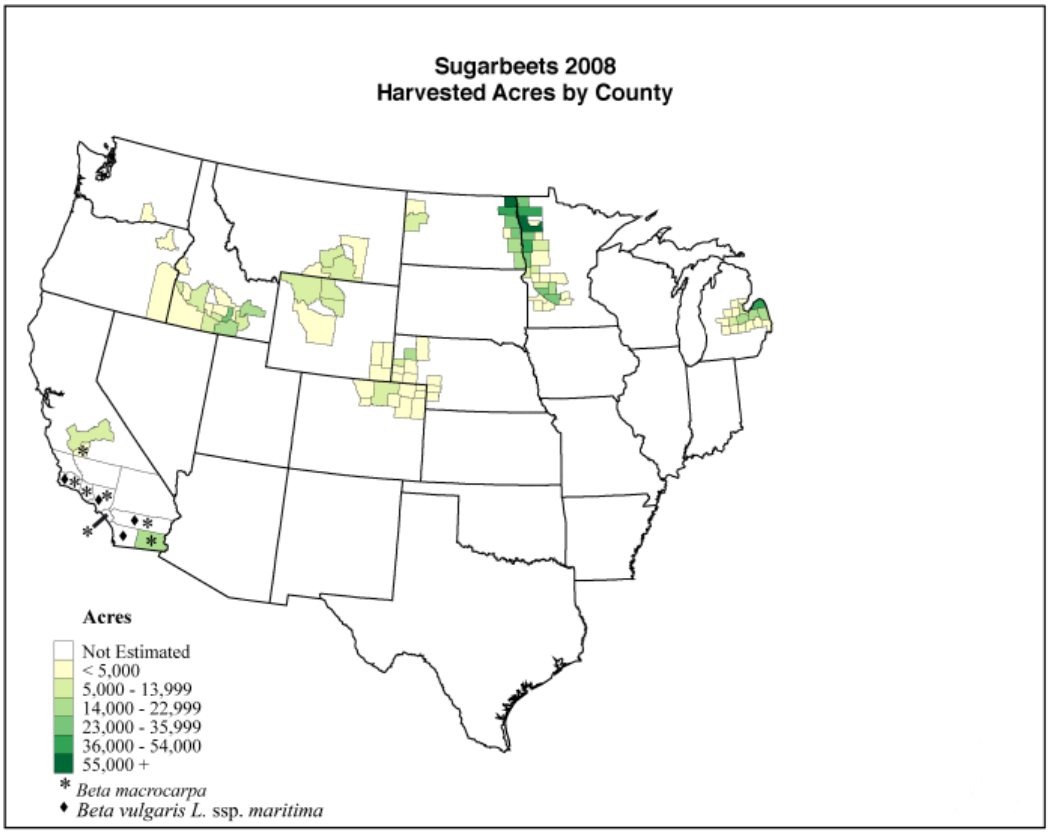

Figure 1. Map of 2008 U.S. sugar beet harvested acreage by county. Asterisk and diamond denote county distribution of the wild beet species *Beta macrocarpa* and *Beta vulgaris* ssp. *maritima,* respectively. The Willamette Valley, OR is also shown. *Beta vulgaris* ssp. *maritima* also occurs in New Jersey, a non-sugar beet producing state, not shown. Adapted from USDA-NASS (http://www.nass.usda.gov/Charts_and_Maps/Crops_County/pdf/SU-HA08-RGBChor.pdf) and from (http://www.oregon.gov/ODA/regions.shtml).

One hectare = 2.471 acres.

Chapter 2

Analysis of U.S. Genetically Engineered Crop Regulation and Litigation

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The commercial potential of genetically engineered (GE) crops has not been fully realized in the United States. Over the past decade, environmental litigation dramatically affected the pace of GE crop development, testing and deregulation. The United States Animal and Plant Health Inspection Service (APHIS) regulates GE organisms that may pose a risk to plant or animal health. However, recent litigation initiated by nongovernmental organizations such as the Center for Food Safety and the International Center for Technology Assessment has exposed APHIS's vulnerability to lawsuits under the National Environmental Policy Act (NEPA) for failing to assess the environmental risks of novel GE crops. In these cases, APHIS committed two types of mistakes. First, APHIS did not differentiate between traditional GE crops whose risks are well characterized and novel GE crops that may raise unique environmental risks and societal issues based on their distinctive biology. Consequently, it did not adequately evaluate the legally defined environmental risks of these novel crops. Second, APHIS did not fully appreciate NEPA's sweeping scope and focus on procedural compliance to ensure transparent and thorough environmental decision making. As a result, APHIS impeded the development and commercialization of GE crops and must take a more defensive posture in the future to deter costly and lengthy NEPA litigation in the case of novel GE crops.

INTRODUCTION

The advent of genetically engineered (GE) plants during the 1980s fascinated the U.S. agronomic and pharmaceutical industries with the complementary prospects of scientific innovation and financial reward. These rosy prospects were reflected in the increasing number of GE crop field tests that the U.S. Animal and Plant Health Inspection Service (APHIS), a division of the USDA, approved each year. The number of annual field tests exponentially grew from 4 in 1985, to 711 by 1995 and peaked in 2002 with 1,194 (Fig. 1). While the new millennia initially held much promise for the plant biotechnology industry, the first decade in the 21st century revealed a different story. New GE crops in the biotechnology pipeline slowed to a trickle by 2010, the first year since 1996 in which approved field tests dipped below 700 (Fig. 1). Furthermore, only two GE crops were deregulated in the United States in 2010 despite over 20 pending petitions (APHIS, 2011a).

Litigation slowed the development, testing, and commercialization of GE crops in the United States. Nongovernmental organizations such as the Center for Food Safety (CFS) and its sister organization, International Center for Technology Assessment (ICTA), filed several lawsuits under the National Environmental Policy Act (NEPA) contesting the adequacy of APHIS's regulation of several GE crops. Consequently, APHIS has been entangled in litigation since 2003. We hypothesize that APHIS impeded biotechnology development and commercialization by omissions in regulatory oversight that left them susceptible to environmental litigation filed by non-governmental organizations. After describing the laws and regulations affecting GE crops, this paper

will analyze APHIS's scientific and legal decision making within the context of recent legal cases brought under NEPA. We will evaluate whether APHIS: (i) complied with the procedural dictates of U.S. environmental law; (ii) recognized environmental risks of novel GE crops as defined by NEPA; and (iii) learned over time from its mistakes. Subsequently, we will explain how the proliferation of litigation has had the unintended effect of driving APHIS to avoid regulation of specific GE crops by using a loophole. Responsible regulation of GE crops based on sound scientific information and conformance with the law should be the goal of APHIS.

OVERVIEW OF GENETICALLY ENGINEERED CROP REGULATION IN THE UNITED STATES

During President Ronald Reagan's administration, proponents of U.S. biotechnology discouraged the creation of a new stand-alone agency to regulate GE crops in 1986 (Schurman and Munro, 2010). Instead, the Coordinated Framework for the Regulation of Biotechnology was developed, which required the cooperation of three federal agencies to regulate plant biotechnology under preexisting statutes (Office of Science and Technology Policy, 1986, 51 FR 23302). The APHIS regulates the planting of GE crops under the Plant Protection Act (2011, 7 U.S.C. §7701 et seq.).The U.S. Food and Drug Administration oversees food and animal feed safety aspects of GE crops (Office of Science and Technology Policy, 1986, 51 FR 23302). The USEPA assumes primary responsibility for crops engineered to produce pesticidal substances such as the *Bacillus thuriengiensis* (*Bt*) pesticidal protein (USEPA, 2011).

The regulations promulgated by APHIS restrict the introduction of GE crops into the environment (APHIS, 2011b). Genetic engineering triggers regulation "if the donor organism, recipient organism, or vector or vector agent" meets the plant pest definition and if one or more of them are specifically listed as plant pests (APHIS, 2011b, 7 C.F.R. §340.1). An organism may also be regulated if the APHIS administrator believes the organism to be a plant pest (APHIS, 2011b). The APHIS primarily regulates the field testing and deregulation of GE crops.

Under APHIS regulations, the field testing of GE crops is called a "release into the environment" (APHIS, 2011b, 7 C.F.R. §340.1). This label is somewhat of a misnomer, because the "release" is largely confined and controlled. Field testing is a precursor to commercialization and entails growing the GE crop across many environments to evaluate crop performance. Monsanto, Pioneer Hi-Bred, and Syngenta are responsible for the majority of APHIS approved field trials (Information Systems for Biotechnology, 2011a).

Currently, APHIS uses two methods to approve field testing. The first method, called "notification," is a streamlined procedure in which the applicant informs APHIS of its intent to conduct a field test (APHIS, 2011b). The applicant provides information on the plant's taxonomy, its phenotype, the genetic material incorporated into the genome, and the method of gene introduction. In addition, the applicant must provide the location and dates of the trial. The notification will be "acknowledged" (approved) within 30 days only if: (i) the plant species is not a noxious weed, (ii) the genetic material is "stably integrated" into the host genome, (iii) the integrated genetic material does not cause plant, animal or human disease or infection, (iv) the genetic material does not encode

pharmaceutical or industrial products, and (v) the genetic material does not encode toxic products that may be consumed by nontarget organisms (APHIS, 2011b). The applicant must agree to meet performance standards for growing the crop in a confined field setting including precautions to prevent the GE crop from persisting in the environment after the trial's conclusion. Over 99% of field tests are performed under this streamlined 30-day procedure (OSTP and CEQ, 2001).

Genetically engineered crops that do not meet the criteria for the notification system must use the more thorough permit system for field tests (APHIS, 2011b). For example, plants producing biopharmaceuticals such as potential vaccines are required to obtain a permit. The permit system emphasizes confinement safeguards to prevent GE crop dissemination. Detailed information must be provided to APHIS including the following nonexhaustive list: (i) the identity and collection location of the donor organisms, the recipient organisms, the vector, and vector agents, (ii) expression of the genetic material and how that expression differs in a non-engineered organism, (iii) molecular biology of the system, (iv) experimental design of the field test, (v) description of the safeguards used to prevent commingling or environmental release, and (vi) description of the plant's final disposition (APHIS, 2011b). In theory, the permit process should be complete within 120 d but in practice can take much longer (Strauss et al., 2010).

After a crop has undergone extensive field testing, the technology owner may petition for deregulation. The crop may be wholly or partially deregulated or else APHIS may deny the petition (*Geertson Seed Farms v. Johanns*, 570 F.3d 1130 (9th Cir. 2009)). Complete deregulation is the path to commercialization by which a crop is removed from

APHIS's regulatory oversight and may be grown like any conventional crop. Under partial deregulation, APHIS may allow deregulation with specific conditions, for example production allowed only in certain geographic areas and prohibited in others. A denial of the deregulation petition means that commercial production is prohibited but field testing may continue under either the permit or notification system. The deregulation petition is intended to justify why the plant should not be regulated as a plant pest based on a complete description of the crop's molecular biology, the genotypic differences between engineered and non-engineered organisms, the GE organism's phenotype, and field test reports (APHIS, 2011b). Public comments on the petition for deregulation are also solicited by publication in the Federal Register before the deregulation decision (APHIS, 2011b).

NATIONAL ENVIRONMENTAL POLICY ACT OF 1969

Genetically engineered crop field testing and deregulation may trigger environmental review under NEPA. This is in addition to APHIS's regulatory responsibilities under the Plant Protection Act, 2011, 7 U.S.C. §7701 et seq. In its introduction, NEPA expressly states that its purpose is to forge "a national policy which will encourage productive and enjoyable harmony between man and his environment" (NEPA, 2010, 42 U.S.C.S. §4321). Under this statute, federal executive agencies must evaluate and document the environmental impact of "major" agency actions that "significantly [affect] the quality of the human environment" (NEPA, 2010, 42 U.S.C.S. §4332). An action's significance depends upon its context and intensity (Council on Environmental Quality, 2010, 40 C.F.R. §1508.27). The human environment is broadly

defined to include the natural and physical environment, people's relationship with the environment, and closely interrelated social or economic effects (Council on Environmental Quality, 2010, 40 C.F.R. §1508.14).

Routine agency actions are categorically excluded from NEPA analysis. For example, permits or notifications for "confined field releases" are categorically excluded, because "the means through which adverse environmental impacts may be avoided or minimized have actually been built right into the actions themselves" (APHIS, 2011c, 7 C.F.R. §372.5(c)). In the case of notifications for field testing, performance standards prevent commingling with non-regulated plant materials and persistence of the regulated plants or offspring in the environment (APHIS, 2011b). Permit applications must contain descriptions of proposed confinement safeguards and be approved by APHIS (APHIS, 2011b). While notifications and permits fall under the categorical exclusion, the NEPA analysis is not complete. An *exception* to the categorical exclusion exists for confined field releases "that involve new species or organisms or novel modifications that raise new issues" (APHIS, 2011c, 7 C.F.R. §372.5(d)(4)). Unfortunately, what constitutes a new or novel species, organism, modification or issue is not specifically defined and has led to controversy and litigation. Field tests that raise new issues must undergo an environmental assessment.

Genetically engineered crop deregulation is usually subject to more scrutiny than field testing. First, APHIS prepares a concise document called an environmental assessment. If the environmental assessment determines that deregulation of a specific crop fails to rise to the threshold of significantly affecting the human environment, then a Finding of No Significant Impact (FONSI) is prepared and the deregulation may proceed

(Council on Environmental Quality, 2010, 40 C.F.R. §1501.4). Conversely, if the environmental assessment determines that deregulation may have a significant environmental impact, then an environmental impact statement is prepared (CEQ, 2010). Environmental impact statements are costly and time-consuming. A complete environmental impact statement can cost over US$1 million as opposed to $60,000 to $80,000 for APHIS to draft an environmental assessment (APHIS, 2011d). Many federal agencies attempt to conserve their scarce resources by preparing concise environmental assessments and thereby avoid environmental impact statements. The number of environmental impact statements by federal agencies has steeply declined over the years and environmental assessments outnumber environmental impact statements approximately 50,000 to 500 (CEQ, 1997).

Federal agencies can avoid preparing NEPA-mandated environmental impact statements by preparing mitigated FONSIs in conjunction with their environmental assessments. A mitigated FONSI is prepared when an agency knows that a proposed action approaches the threshold of becoming a significant environmental impact. The agency stoops under the perceived threshold by including mitigation factors (Karkkainen, 2002). While the mitigated FONSI may sound devious, it is a well-accepted tactic (*Cabinet Mountains Wilderness v. Peterson*, 1982). To prepare a mitigated FONSI, the agency must understand the direct and indirect consequences of its proposed action so that it may determine the significant environmental impact threshold. The mitigating factors then modify the proposed action so that it no longer threatens to degrade the human environment. The environmental assessment and the mitigated FONSI are published so that the decision-making process is transparent. An agency's decision to

prepare an environmental assessment and not an environmental impact may be challenged in court. The court can overturn the agency's decision, if it was arbitrary or capricious (Administrative Procedures Act, 2011, 5 U.S.C. §706(2)(A)).

FIELD TESTING LITIGATION

Two lawsuits challenging APHIS's decisions to allow field testing of controversial crops have been litigated. While the courts focused on the procedural requirements of NEPA categorical exclusions, they hinted at more substantive, scientific lapses. In *Center for Food Safety v. Johanns*, 451 F.Supp.2d 1165 (D. Haw. 2006), APHIS granted four permits to allow Prodigene, Monsanto, and two other companies to conduct field testing of human biopharmaceutical crops from 2001 to 2003 in Hawaii. The permit holders grew GE corn (*Zea mays* L.) and sugarcane (*Saccharum* spp.) that produced experimental hormones, vaccines for the human immunodeficiency virus or Hepatitis B virus, or cancer-fighting agents. While the judicial opinion is deliberately short on crop specifics due to confidentiality concerns, it appears that all field tests were confined and completed without adverse environmental effects. After the conclusion of field testing, Center for Food Safety (CFS) and other non-governmental organizations filed suit against USDA Secretary Mike Johanns and the head of APHIS in late 2003 for issuing permits in violation of NEPA. The CFS alleged that APHIS should have prepared an environmental assessment or environmental impact statement before issuing the permits to evaluate environmental risks such as: (i) gene flow from biopharmaceuticals to food crops, (ii) commingling of harvested biopharmaceutical products with food crops, and (iii) consumption by animals. In defense of its actions, APHIS asserted that the field test

permits fell under NEPA's categorical exclusion. While its sparse administrative record stated that the field tests were "confined", APHIS's documentation failed to expressly state that it was invoking the categorical exclusion (*Center for Food Safety v. Johanns*, 451 F.Supp. 2d 1165, (D. Haw. 2006)). Although APHIS argued that it substantively complied with the categorical exclusion requirements by ensuring that the field tests were confined and controlled, the court was not swayed. It concluded that APHIS's decision to issue the permits without documenting its environmental decision processes was a NEPA violation. Furthermore, the judge was troubled by APHIS's failure to evaluate whether the exception to the categorical exclusion for "novel modifications" applied (*Center for Food Safety v. Johanns*, 451 F.Supp. 2d 1165, (D. Haw. 2006)).

In its decision to allow field testing of a biopharmaceutical crop, APHIS violated more than one statute. The court ruled that APHIS violated the Endangered Species Act, 2010, 16 U.S.C.S. §1531 et seq., by failing to obtain a list of Hawaiian endangered and threatened species before issuing the permits. Hawaii is home to 329 federally endangered and threatened animal and plant species or almost 25% of all federally listed species (*Center for Food Safety v. Johanns*, 451 F.Supp. 2d 1165, (D. Haw. 2006)). As its defense, APHIS asserted that no endangered or threatened species were harmed during the field trials. However, this after-the-fact defense was inadequate and the court held that APHIS violated the Endangered Species Act by failing to comply with its procedural requirements.

International Center for Technology Assessment v. Johanns , 473 F.Supp.2d 9 (D.D.C. 2007), shows that APHIS's procedural omissions were not a singular occurrence. In this case, CFS and its sister organization, ICTA, sued APHIS for approving Scotts

Miracle-Gro Company's notification of field testing of GE turf grasses in Oregon and other states from May 2002 through July 2003. Scotts GE creeping bentgrass (*Agrostis stolonifera* L.) is engineered to be tolerant of the non-selective herbicide, glyphosate, the active ingredient in Roundup. The CFS and ICTA claimed that APHIS violated NEPA when it allowed field testing to proceed under the notification system without preparing either an environmental assessment or environmental impact statement. As in *Center for Food Safety v. Johanns*, APHIS alleged that it allowed field testing under the categorical exclusion even though the administrative record was devoid of such a determination. In direct contrast to *Center for Food Safety v. Johanns*, the Court held that APHIS was *not* required to explicitly invoke the categorical exclusion, because field testing of GE crops falls squarely within this exclusion (*International Center for Technology Assessment v. Johanns* , 473 F.Supp.2d 9 (D.D.C. 2007)). Nevertheless, the Court concluded that APHIS violated NEPA, because it failed to make a determination as to whether the crop involved new species or novel modifications or issues that would negate the categorical exclusion.

On the surface, the two cases highlight procedural omissions. Under NEPA analysis, the question before the court is whether APHIS followed proper procedures in reaching a conclusion as opposed to whether the agency reached the right environmental conclusion. The court concluded that APHIS failed to document potential environmental concerns before approving field tests. Inattentiveness in NEPA documentation may be explained by the lack of GE crop litigation prior to 2003. This recordkeeping neglect proved deleterious, because NEPA is a procedural as opposed to substantive statute and requires an agency to conduct informed decision making on environmental matters

(*Vermont Yankee Nuclear Power Corp. v. National Resources Defense Council, Inc*., 435 U.S. (1978)).

In hindsight, APHIS should have compiled a more thorough administrative record. Its attorneys could have designed an internal checklist to prompt regulators to document whether they were invoking the categorical exclusion and whether the exception to the exclusion had been considered. The checklist could also have ensured that APHIS obtained a list of endangered and threatened species and considered the environmental impact on those species. These simple procedural steps would create a transparent record and could deter future litigation.

While these two cases hinged on procedural failures, they also hinted at substantive, scientific issues. APHIS failed to recognize that both cases involved novel species, modifications or issues that were a significant departure from traditional GE agronomic crops such as *Bt* corn and cotton (*Gossypium hirsutum* L.) or glyphosate-tolerant (GT) soybeans. Incorporating a biopharmaceutical trait such as an experimental vaccine into field crops may pose human and wildlife consumption risks. The potential for gene flow, for accidental commingling with food crops, and for persistence in the environment should have been documented and addressed in an environmental assessment.

Likewise, GT creeping bentgrass also raised equally novel issues, because the underlying turfgrass species is more likely to persist and aggressively spread beyond golf courses and managed landscapes compared to traditional GE crops. Creeping bentgrass is a perennial and is extremely cold-hardy even in northern regions of the U.S. (Warnke, 2003); therefore, it is more likely to survive than annuals after cessation of field trials. In addition, the species is considered to be a weed through much of the United States

(Bryson and DeFelice, 2009; Stubbendieck et al., 1994; Whitson et al., 1996). Its weediness stems from the vigorous vegetative spread of its stolons (Bryson and DeFelice, 2009) and through sexual reproduction via seed set. This European weedy species has been introduced throughout the United States (National Resources Conservation Service, 2011). Like most grasses, creeping bentgrass is wind-pollinated (Watrud et al., 2004), which can facilitate gene flow over large distances. These novel issues were not trivial concerns, because gene flow did occur during Scotts' field testing. A USEPA study published during the midst of litigation confirmed transgenic pollination of established wild and sentinel (deliberately planted) populations of non-transgenic creeping bentgrass plants 8 and 21 km distant respectively from Scotts' field plots (Watrud et al., 2004). In addition, Watrud et al., 2004 found evidence of transgenic hybridization with redtop (*Agrostis gigantea* Roth). Thus, the introduction of glyphosate tolerance into this aggressive species makes glyphosate ineffective in controlling this transgenic weed and its hybrids if it spreads to rangelands and other natural ecosystems.

Learning from its mistakes, APHIS has now recognized that the testing of perennial GE crops may raise novel issues. In 2008, APHIS announced that field tests that last in excess of one year must seek approval through the permit process and not through the notification system (APHIS, 2008). This will subject perennial crops to more scrutiny and may strengthen the scientific decision-making process.

The above two cases had far-reaching ramifications beyond the crops involved in the litigation. The number of field tests began falling in 2003, the year *Center for Food Safety v. Johanns* was filed.

By 2010, the number of approved field tests dipped below 700 (Fig. 1) as investment was diverted. The cases had an even more dramatic effect upon CFS and its sister organization, ICTA. Judicial victory before federal district courts in Hawaii and Northern California entailed a substantial economic reward for CFS and ICTA. The Equal Access to Justice Act, 2011, 28 U.S.C.S. §2412, provides for an award of attorneys' fees and costs in successful litigation against government agencies. In the biopharmaceutical and creeping bentgrass cases, CFS and ICTA were awarded $574,617.43 and $85,141.24 respectively (*Center for Food Safety v. Johanns*, 2007 U.S. Dist. LEXIS 77438 (D. Haw. 2007); *International Center for Technology Assessment v. Vilsack*, 602 F.Supp.2d 228 (D.D.C. 2008)). Success emboldened the two organizations and provided a financial incentive to continue NEPA litigation with respect to deregulation cases.

DEREGULATION CASES

Deregulation decisions significantly differ from field testing permits and notifications due to the potential widespread and permanent ramifications of approving a GE crop for commercial production. When evaluating a GE crop for deregulation, APHIS must prepare an environmental assessment and then decide whether the findings merit an environmental impact statement. In the following two cases, APHIS prepared environmental assessments and concluded there were no significant environmental impacts to justify preparing environmental impact statements.

The judiciary may review an agency's decision to forego preparation of an environmental impact statement under the following criteria: "(1) whether the agency

took a 'hard look' at the problem; (2) whether the agency identified the relevant areas of environmental concern; (3) as to the problems studied and identified, whether the agency made a convincing case that the impact was insignificant . . ." (*Cabinet Mountains Wilderness v. Peterson*, 1982, 685 F.2d at 682). The definition of a "hard look" is rather subjective and is decided on a case by case basis; however, if an agency can show that it gathered and thoroughly evaluated all relevant information, then the judiciary cannot overturn the agency's environmental decision (*Kleppe v. Sierra Club*, 427 U.S. 390 (1976)). *Foundation for North American Wild Sheep v. U.S.D.A.,* 681 F.2d 1172 (9th Cir. 1982), illustrates an agency decision that did not satisfy the "hard look" test. Based upon its environmental assessment and a FONSI, the U.S. Forest Service issued a permit to a tungsten mine to reconstruct and use a mountain road that passed through prime desert bighorn sheep (*Ovis canadensis nelsoni* (Merr.)) habitat in a national forest. In reviewing the Forest Service's decision not to prepare an environmental impact statement, the court concluded that it failed to take a "hard look" because the agency's environmental assessment omitted any estimate of potential traffic on the road through this sensitive habitat. Consequently, the failure to consider key factors meant that the Forest Service could not make an informed decision (*Foundation for North American Wild Sheep*, 681 F.2d 1172 (9th Cir. 1982). While the facts are different, this same focus on the agency taking a "hard look" permeates the following two cases.

In *Geertson Seed Farms v. Johanns*, 2007 WL 518624 (N.D. Cal. 2007), CFS, Sierra Club and several organic farm organizations sued the USDA Secretary and APHIS for deregulating glyphosate-tolerant ("GT") alfalfa (*Medicago sativa* L.) without preparing an environmental impact statement. Alfalfa is the nation's fourth largest

agronomic crop by land area (APHIS, 2005), and differs significantly from traditional agronomic crops in its life cycle, reproductive biology and production methods. Glyphosate-tolerant alfalfa is a pioneering GE crop because it is one of the first bee-pollinated, perennial agronomic crops to be deregulated by APHIS. Glyphosate tolerance was welcomed by some alfalfa farmers because it provided a flexible weed control option after the perennial crop was established. The lawsuit objected to deregulation on the basis that APHIS didn't adequately address the risk of pollen flow from flowering GT alfalfa fields to neighboring organic or conventional alfalfa seed fields (*Geertson Seed Farms v. Johanns*, 2007 WL 518624 (N.D. Cal. 2007)). If a bee acquires GT alfalfa pollen and pollinates an organic alfalfa plant in a nearby field, then the resulting seeds may carry the GT gene. The USDA-administered National Organic Program prohibits organic growers from utilizing genetic engineering to produce a crop (National Organic Program, 2011, 7 C.F.R. §205). In its concise environmental assessment, APHIS briefly acknowledged that alfalfa is bee-pollinated but did not identify the primary species and the distance that the insects travel. Instead, APHIS asserted that organic farmers could plant buffer zones to prevent cross-pollination between transgenic and organic seed crops (APHIS, 2005). Additionally, APHIS stated that the USDA's National Organic Standards do not require testing for transgene presence in order for them to be certified as organic (APHIS, 2005). The U.S. District Court for the Northern District of California was not persuaded and stated: "the significant impact that requires the preparation of an [environmental impact statement] is the possibility that the deregulation of Roundup Ready alfalfa will degrade the human environment by eliminating a farmer's choice to grow non-genetically engineered alfalfa and a consumer's choice to consume such food" (*Geertson Seed Farms*

v. Johanns, 2007 WL 518624, p. 9 (N.D. Cal. 2007)). Thus the court concluded that APHIS failed to comply with NEPA's requirements to take a "hard look" at the potential environmental impacts of its decision. The court also ruled that APHIS further violated NEPA by not considering the cumulative impact of increased glyphosate use on the development of GT weeds (*Geertson Seed Farms v. Johanns*, 2007 WL 518624 (N.D. Cal. 2007)). In the subsequent remedies phase of the litigation, Monsanto was allowed to intervene and participate in the proceedings. Nonetheless, the Court voided the deregulation decision, ordered APHIS to prepare an environmental impact statement and enjoined or prohibited further planting of GT alfalfa pending the completion of the environmental impact statement (*Geertson Seed Farms v. Johanns*, 2007 WL 518624 (N.D. Cal. 2007)).

Monsanto appealed the permanent injunction prohibiting the planting of GT alfalfa to the 9th Circuit Court of Appeals and then to the Supreme Court of the United States. The Supreme Court of the United States decided only the narrow issue of the proper remedies after a NEPA violation. The Supreme Court affirmed the lower court's right to void the agency's deregulation decision in the event of a NEPA violation; thus returning GT alfalfa to a regulated status under APHIS's jurisdiction (*Monsanto Co. v. Geertson Seed Farms*, 130 S.Ct. 2743 (U.S. 2010)). In addition, the Supreme Court overturned the lower court's broad injunction prohibiting *all* further planting pending preparation of the environmental impact statement. The Supreme Court could foresee conditions under which APHIS could properly issue an interim permit or a partial deregulation to plant under regulated and confined conditions subject to NEPA requirements while it was preparing an environmental impact statement (*Monsanto v. Geertson*, 130 S.Ct. 2743

(U.S. 2010)). This important ruling will be revisited in the context of the sugar beet (*Beta vulgaris* L. subsp. *vulgaris* L.) litigation.

In light of the fact that APHIS had advance notice of opposition, its failure to comprehensively address the concerns of the organic industry is troubling because they had advance notice of opposition. During the public comment period after the draft environmental assessment was published, APHIS received 663 public comments of which 520 were negative (*Geertson Seed Farms v. Johanns*, 2007 WL 518624 (N.D. Cal. 2007)). Therefore, APHIS and the USDA were aware of the opposition and of the novel issues raised by this crop. The USDA could have attempted to work with its diverse constituencies to mitigate environmental concerns. If stakeholder negotiations failed, APHIS and the USDA could have produced a more thorough and convincing finalized environmental assessment in an attempt to avoid litigation and adverse judicial precedent.

The environmental assessment prepared by APHIS (APHIS, 2005) in *Geertson Seed Farms v. Johanns* is not an example of the "hard look" doctrine as required by NEPA. First, the extremely concise environmental assessment utterly failed to evaluate the potential for insect-mediated gene flow from GE alfalfa seed fields to adjacent organic alfalfa seed fields. Three types of bees with different roaming ranges are primarily used to pollinate alfalfa flowers depending upon geographic seed region: European honey bees (*Apis mellifera* L.) in California, leafcutter bees (*Megachile rotundata* Fabricious) in the Pacific Northwest, and alkali bees (*Nomia melanderi* Cockerell), a small niche species in southern Washington (Van Deynze et al., 2008). In a leafcutter bee study, gene flow from GT alfalfa to nontransgenic alfalfa decreased rapidly with distance to less than 1% at 305 m and to 0.003% at 805 m (Fitzpatrick et al., 2003).

Honey bee cross-pollination was confirmed at the rate of 1.49% at 274 m, decreased to 0.20% at 1524 m and was still detectable at low frequencies (0.06%) at 4.07 km (Teuber et al., 2004). Considering that honey bees forage over large distances, organic alfalfa seed farmers would have difficulty in preventing gene flow from neighboring GT alfalfa fields.

Second, APHIS didn't identify that GT alfalfa produced for hay as opposed to seed may be a transgenic pollen source. Alfalfa hay is produced on 21,000,000 acres in the United States as of 2008 (NASS, 2011). While most farmers harvest alfalfa hay when the crop is just beginning to flower to ensure highest forage quality, this can vary depending upon weather delays and farmer practices. The environmental assessment did not address the likelihood of delayed harvesting resulting in flowering and pollen release; the hay crop could flower and produce transgenic pollen available for insect delivery. Therefore, both GE alfalfa seed and hay production raise important environmental issues.

In failing to grapple with the potential for transgenic gene flow to organic seed crops and to make a convincing case that the impacts were insignificant, APHIS left itself vulnerable to judicial interpretation of NEPA's sweeping scope and the meaning of an action that "significantly affect[s] the quality of the human environment" (NEPA, 2010, 42 U.S.C.S. §4332). It is easy to understand that federal actions that affect national parks, forests and wildlife are protected under the NEPA umbrella. These kinds of actions have a strong nexus to the physical environment and to unique ecosystems. However, *Geertson Seed Farms v. Johanns* defines cross-pollination between transgenic and organic seed crops as degradation of the human environment. Certainly, whether APHIS should have foreseen NEPA's broad reach is debatable. For example, cross-pollination has always

occurred between neighboring crops. This is why we have seed organizations to establish isolation distances to ensure seed purity. On the other hand, careful study of NEPA precedent shows how broadly the human environment is legally defined. In *Hanley v. Kleindienst,* 471 F.2d 823 (2nd Cir. 1972), Manhattan businesses and residents filed a NEPA lawsuit against the General Services Administration objecting to the building of a federal detention center next to the federal courthouse in their neighborhood. The detention center was to house individuals awaiting trial, serving short sentences, or engaged in a work-release program. Plaintiffs argued that the General Services Administration should have prepared an environmental impact statement because the project significantly affected the quality of the human environment in Manhattan—namely the project had the potential for increased crime and riots. The United States Court of Appeals for the Second Circuit agreed with Plaintiffs and remanded the case for further inquiry into these issues (*Hanley v. Kleindienst*, 471 F.2d 823 (2nd Cir. 1972)). While *Hanley v. Kleindienst* is not directly analogous to the APHIS cases, it is still important because it shows NEPA's extensive reach. The concrete jungle of Manhattan with all of its traffic and noise is still considered a human environment to be protected. If the judiciary can label a busy urban center as part of the human environment, then it is not much of a stretch to label an agricultural ecosystem as a protected environment.

Now APHIS must live with the negative judicial precedent that *Geertson Seed Farms v. Johanns* has created. The District Court for the Northern District of California has equated *any* amount of gene flow to organic crops with an incurable infection (*Geertson Seed Farms v. Johanns,* 2007 WL 518624 (N.D. Cal. 2007)). Consequently, the District Court for the Northern District of California will now be a magnet for anti-

biotechnology cases. The semantic inaccuracy of referring to gene flow as an infection even continues in the Supreme Court's opinion (*Monsanto Co. v. Geertson Seed Farms*, 2010). It is readily apparent that the judiciary still does not understand the nature of genetic engineering.

The alfalfa saga will continue. Subsequently, APHIS prepared a voluminous environmental impact statement and deregulated GT alfalfa a second time in February, 2011. The CFS quickly filed suit to contest the adequacy of the environmental impact statement. The District Court for the Northern District of California ruled in favor of APHIS and refused to vacate the deregulation (*Center for Food Safety v. Vilsack*, 2012 U.S. Dist. LEXIS 1214 (N.D. Cal. 2012). It is unknown whether CFS will appeal the decision.

After *Geertson Seed Farms v. Johanns*, CFS targeted the deregulation of GT sugar beet (*Beta vulgaris* L.), a source of refined sucrose. This root crop is different from most agronomic crops, because it is a wind-pollinated biennial and its sexually compatible relatives, Swiss chard and table beet (both *Beta vulgaris* L.), are grown for the fresh vegetable market in the U.S. (McGinnis et al., 2010). In *Center for Food Safety v. Vilsack,* 2009 U.S. Dist. LEXIS 86343 (N.D. Cal. 2009), plaintiffs CFS, Sierra Club, and organic seed organizations sued APHIS in the District Court for the Northern District of California for deregulating GT sugar beet without preparing an environmental impact statement to evaluate the risk of transgenic cross-pollination of organic Swiss chard or table beet seed crops in the seed Willamette Valley, Oregon (2009). Sugar beet is not produced organically. With *Geertson Seed Farms v. Johanns* as precedent, the court ruled that APHIS violated NEPA and must prepare an environmental impact statement before

deregulating GT sugar beet (*Center for Food Safety v. Vilsack*, 2009 U.S. Dist. LEXIS 86343 (N.D. Cal. 2009)). The paucity of the administrative record supports the court's conclusion that APHIS violated NEPA. With the exception of a brief mention in the appendix of sugar beet's taxonomic relatives, the main text of the environmental assessment omitted discussion that sugar beet, Swiss chard and table beet are the same species and capable of cross-fertilization (APHIS, 2004).

The environmental impact in *Center for Food Safety v. Vilsack* appears to be more geographically isolated than in the case of GT alfalfa, because of the distinctive life cycle, production methods, and distribution of sugar beet (McGinnis et al., 2010). Sugar beet is a biennial that produces a sucrose-containing storage root in its first year and then draws on its sugar reserves to flower in its second year, if overwintered in a mild climate (Milford, 2006). Root production must be differentiated from seed production; roots raised for sucrose processing are harvested in the first year before they can flower. Seed production is geographically separate from root production and predominantly takes place in the Willamette Valley, Oregon (McGinnis et al., 2010). Seed production takes place in two stages. First, seeds are planted in the fall and the plant is grown until it is vernalized in mid-winter (steckling production). In the second stage, stecklings are lifted in February, transplanted to final field spacing, flower in June and set seed over the summer. Organic Swiss chard and table beet seed crops follow a similar schedule and thus, there is the potential for gene flow in the Willamette Valley in the absence of significant isolation distances. Despite the geographically limited nature of gene flow, the Court vacated APHIS's deregulation of GT sugar beet on 13 Aug. 2010 (*Center for Food Safety v. Vilsack,* 734 F.Supp.2d 948 (N.D. Cal. 2010)). Thus, both root and seed

production were returned to regulated status nationwide pending the completion of an environmental impact statement. It is notable that the court did not permanently enjoin field releases under a permit or partial deregulation due to Supreme Court precedent.

In hindsight, APHIS could have avoided the sugar beet litigation and the costly preparation of an environmental impact statement if it had recognized the potential for unwanted gene flow. Based upon sugar beet seed production's geographic isolation, APHIS could have implemented a mitigated FONSI with its environmental assessment. The mitigation measures could have significantly decreased the potential for gene flow to the one or two affected Swiss chard and table beet producers by including scientifically supported isolation distances between transgenic and organic seed crops in the Willamette Valley (McGinnis et al., 2010). If it had adopted the mitigated FONSI strategy, APHIS might have made better environmental decisions and avoided litigation.

THE UNITED STATES ANIMAL AND PLANT HEALTH INSPECTION SERVICE: GOING BACK TO THE BOOKS

The cases discussed above schooled APHIS in U.S. environmental litigation. It is apparent that APHIS needed to nimbly adjust its regulation and its legal strategy. The sugar beet sequels to *Center for Food Safety v. Vilsack* give us an opportunity to evaluate APHIS's ability to adapt to several years of litigation. On 3 Sept. 2010, a mere 21 days after the Court reversed the GT sugar beet deregulation, APHIS granted field permits to four sugar beet seed companies to commence steckling production in the Willamette Valley (71 acres) and in Arizona (455 acres) (Information Systems for Biotechnology, 2011b). The purpose of these permits was to produce stecklings for commercial seed

production trials. These permits were granted under the categorical exclusion for field testing permits and no environmental assessment or impact statement was prepared. The permits only authorized steckling production and strictly prohibited flowering. It was anticipated that a second permit or some other action on the part of APHIS would be required for transplanting the vernalized stecklings and allowing flowering and seed set.

At first glance, APHIS's actions in granting these permits appeared foolhardy coming on the heels of *Center for Food Safety v. Vilsack*. However in hindsight, these permits were APHIS's attempt to test the waters by using U.S. Supreme Court precedent from *Monsanto Co. v. Geertson Seed Farms* (the alfalfa appeal) to undertake temporary or interim regulation while it was preparing the court-ordered environmental impact statement for GT sugar beet. Not surprisingly, CFS immediately filed a second NEPA suit on 9 Sept. 2010 with the District Court for the Northern District of California and requested an injunction for the destruction of the stecklings (*Center for Food Safety v. Vilsack*, 2010 U.S. Dist. LEXIS 141390 (N.D. Cal. 2010)). Despite APHIS's arguments that these field testing permits prohibited gene flow by not allowing these stecklings to flower, the Court ordered their destruction in the 30 Nov. 2010 order (*Center for Food Safety v. Vilsack*, 753 F.Supp.2d 1051 (N.D. Cal. 2010)). The Court ruled that it was impermissible to segment seed production into two stages (vernalized steckling and flowering/seed set) to evade environmental assessment (*Center for Food Safety v. Vilsack*, 2010 U.S. Dist. LEXIS 141390 (N.D. Cal. 2010)).

The sugar beet industry and APHIS were not done testing the judicial waters. The case was appealed to the United States Court of Appeals for the Ninth Circuit and the lower court's decision was reversed on 25 Feb. 2011 as an abuse of discretion (*Center for*

Food Safety v. Vilsack, 636 F.3d 1166 (9th Cir. 2011)). In its legal analysis, the Ninth Circuit found that irreparable harm to the environment through gene flow was almost nonexistent because the permits did not allow flowering (*Center for Food Safety v. Vilsack*, 636 F.3d 1166 (9th Cir. 2011)). The Ninth Circuit did not have a problem with segmenting seed production into two stages because CFS could bring another NEPA lawsuit, if APHIS approved a permit allowing for flowering of the stecklings without proper environmental review.

On 4 Feb. 2011, APHIS announced its partial deregulation of GT sugar beet after publishing an environmental assessment (APHIS, 2011e) and a mitigated FONSI (APHIS 2011f). This is APHIS's first partial deregulation of a GE crop and utilized U.S. Supreme Court precedent from *Monsanto Co. v. Geertson,* 130 S.Ct. 2743 (U.S. 2010), to allow limited interim planting while APHIS completed the environmental impact statement on GT sugar beet deregulation. This partial deregulation allowed farmers to plant sugar beet as a root crop for the 2011 season subject to strict compliance agreements that required: (i) periodic farmer and third-party inspections to insure the removal of bolting (flowering) plants; (ii) reporting within 48 hours to APHIS when bolters are found and the action taken (iii) three years of monitoring for volunteer plants; (iv) a minimum of four year sugar beet crop rotations; (v) extensive record-keeping and training; (vi) containment of trucks hauling beets for processing; and (vii) tertiary containment of seeds in transit (APHIS, 2011g). The partial deregulation also allowed seed production subject to even more stringent conditions including (i) a 6,437 m isolation distance between GT sugar beets and other *Beta vulgaris* seed crops even if the GT sugar beets are male-sterile; (ii) extensive record-keeping; (iii) measures to prevent

commingling of GT sugar beet with non-regulated material; (iv) equipment cleaning and segregation; (v) transport and containment protocols; and (vi) field sanitation and monitoring (APHIS, 2011g).

Consequently on 7 Feb. 2011, sugar beet growers, processors, and seed producers filed suit in the District Court for the District of Columbia asking for a two-pronged declaratory judgment (*Grant v. Vilsack,* File No. 1:11-cv-00308JDB (2011)). The first count alleged that APHIS imposed unreasonable costs and burdens on the sugar beet industry when it required a four-year rotation for root production, onerous bolter reporting requirements, and multiple layers of seed containment as requirements for root production. The four-year rotation on root production was unnecessary to stop gene flow because root production does not geographically overlap with seed production. In addition, the bolter reporting requirement was quite burdensome, because it obligated growers to report bolters to APHIS within 48 hours and to also report the absence of bolters. With respect to seed production, plaintiffs objected to the 6,437 m isolation distance between sugar beet seed crops and other *Beta vulgaris* crops as unnecessary. In its second count, plaintiffs asked for a declaratory judgment from the court ruling that APHIS's partial deregulation, environmental assessment and FONSI were in compliance with NEPA and that CFS could not compel the sugar beet industry to destroy their 2011 GT sugar beet crops that were planted across the country (*Grant v. Vilsack*, File No. 1:11-cv-00308JDB (2011)). While the first count took an adversarial position to APHIS, it was clear that the purpose of this lawsuit was twofold: (i) to strike preemptively to ensure that the District Court for the Northern District of California did not hear the case; and (ii) to obtain a ruling affirming that APHIS's partial deregulation was valid and could not be

overturned by CFS. Concurrently, CFS filed a lawsuit in the District Court for the Northern District of California contesting the partial deregulation in an attempt to venue the case before a sympathetic judge. Both the California and the District of Columbia cases have been consolidated and are being heard in the District of Columbia.

It is uncertain whether APHIS's partial deregulation will be upheld because *Grant v. Vilsack* has not been litigated to a conclusion. However, the case may be moot because the 2011 harvest has drawn to a close and APHIS has just released a draft environmental impact statement recommending that GT sugar beet will once again be deregulated. After evaluating public comment and holding stakeholder meetings, APHIS will then finalize its environmental impact statement.

FORCES IMPACTING REGULATORY MISTAKES

The above sections detail lapses in GE crop regulation and NEPA compliance. Even APHIS tacitly admitted that its NEPA compliance may be subject to improvement in its 7 April 2011 press release stating that it is implementing a pilot project to evaluate new approaches to efficiently generating the necessary environmental documentation to comply with NEPA in deregulation petitions (APHIS, 2011d). The two-year pilot project would explore two mechanisms: (i) the biotechnology petitioner would generate a comprehensive environmental report that would enable APHIS to swiftly generate a draft environmental assessment or environmental impact statement or (ii) the biotechnology petitioner could choose to pay to have the environmental assessment or environmental impact statement prepared by an APHIS-approved third-party contractor.

Several hypotheses may explain why APHIS's environmental decision-making was prone to NEPA legal challenges. First, we believe that APHIS's legal guidance was insufficient during the early years of GE crop regulation. It is also possible that APHIS favored the plant biotechnology industry over the interests of organic producers due to the revolving door between the biotechnology industry and APHIS, a position discussed in depth by Mattera (2004) and Meghani and Kuzma (2010). However, it is also likely that APHIS had grown lax in its NEPA compliance, because its staff was overwhelmed by the flood of notifications, permits and petitions for deregulation during the late 1990s and the beginning years of this century. The agricultural biotechnology boom also coincided with the decreasing power of anti-biotechnology activists in the early part of the first decade (Schurman and Munro, 2010). This simultaneous decrease in public interest and increase in product discovery and development may have lured APHIS into complacency.

However, while the number of activist groups dwindled by 2004 (Schurman and Munro, 2010), APHIS now recognizes that even a single antibiotechnology activist group can block the biotechnology pipeline. A constant through all the cases, is that CFS or its sister organization ICTA was the lead plaintiff. CFS and ICTA have been savvy in their legal strategy. They "forum shopped" by filing their cases in receptive federal district courts such as the Northern District of California and Hawaii. They deliberately depicted transgenic gene flow as an ever spreading infection that will eliminate the availability of organic seeds. Even though this depiction is scientifically inaccurate and misleading, the judicial system bought into the negative semantics.

By filing case after case, they set precedent that even the potential for a minimal amount of cross-pollination of organic crops equals a significant environmental impact. These two organizations have also been economically efficient and self-sustaining. None of their cases go to trial but instead are decided on the basis of much less expensive motion hearings. Furthermore, every time they prevail in a NEPA case, they are awarded a substantial amount in attorneys' fees and costs even though they have in-house counsel. The CFS and ICTA therefore have economic incentives to continue litigation against APHIS and will undoubtedly continue to play a major role in the future unless APHIS addresses the environmental complexities of novel GE crops.

However, CFS and ICTA failed to foresee that their litigation strategy to block the biotechnology pipeline by preventing all transgenic gene flow would backfire. In response to negative precedent, APHIS is trying a new tactic. APHIS decided not to regulate certain GE crops (and thus avoid subsequent environmental litigation) through a rather conspicuous loophole. In 2010, the Scotts Miracle-Gro Company petitioned the USDA to confirm that its GE Kentucky bluegrass (*Poa pratensis* L.) is not subject to USDA regulation. Ordinarily, APHIS would regulate this GE plant under its plant pest authority because the gene of interest, the vector, the 35S promoter and other genetic sequences would come from a listed plant pest such as modified figwort mosaic virus. However, Scotts derived the genetic sequences from non-pest species. The 5-enolpyruvylshikimate-3-phosphate synthase came from *Arabidopsis thaliana* [(L.) Heynh.], the ubiquitin promoter and the actin intron came from *Oryza sativa* (L.), and the alcohol dehydrogenase 3' UTR came from *Zea mays* (L.). The gene introduction was done biolistically without the use of the plant pest *Agrobacterium tumefaciens*. Even

though the genetic sequences did not come from a plant pest, the APHIS administrator still had the discretion to regulate GT Kentucky bluegrass if he had reason to believe that it might be a plant pest. However, APHIS announced in its 1 July 2011 news release that it would not regulate GT Kentucky bluegrass, because none of the organisms that provided material were plant pests. Previously, APHIS declined to regulate two minor crops—a scented geranium (*Pelargonium* spp. (L'He'r. ex Aiton) in 1993 that was grown as a houseplant and a petunia (*Petunia* x*hybrida* (Juss.) in 2003 with altered petal color. These decisions in concert with the GT Kentucky bluegrass announcement are tantamount to publishing a blueprint on how to avoid GE crop regulation and NEPA review if APHIS makes consistent decisions. However, we believe this course of action is unnecessary. We believe that APHIS will not inspire public confidence by exploiting loopholes. Instead, APHIS is better off putting its efforts into efficiently and transparently complying with NEPA by addressing the underlying issues that arise from novel crops and by engaging with stakeholders to implement low level tolerances for transgenic gene flow to organic crops.

CONCLUSION

The Animal and Plant Health Inspection Service's regulation of GE crops has been prone to litigation. The legal cases show that APHIS has had lapses in recognizing environmental risks and in understanding U.S. environmental law. As a consequence, the GE crop pipeline is partially blocked and investments are being diverted to other forms of technology. In the future, APHIS needs to take a more transparent and thorough approach to evaluating the environmental risks of novel crops. We are not advocating more

regulation for routine crops. Furthermore, APHIS must utilize its authority for mitigation of environmental risk as well as understand the legal strategies for complying with U.S. environmental law. Well-informed environmental decision making is essential for minimal litigation, increased stability and the appropriate regulation of GE crops allowing for continued technological advances.

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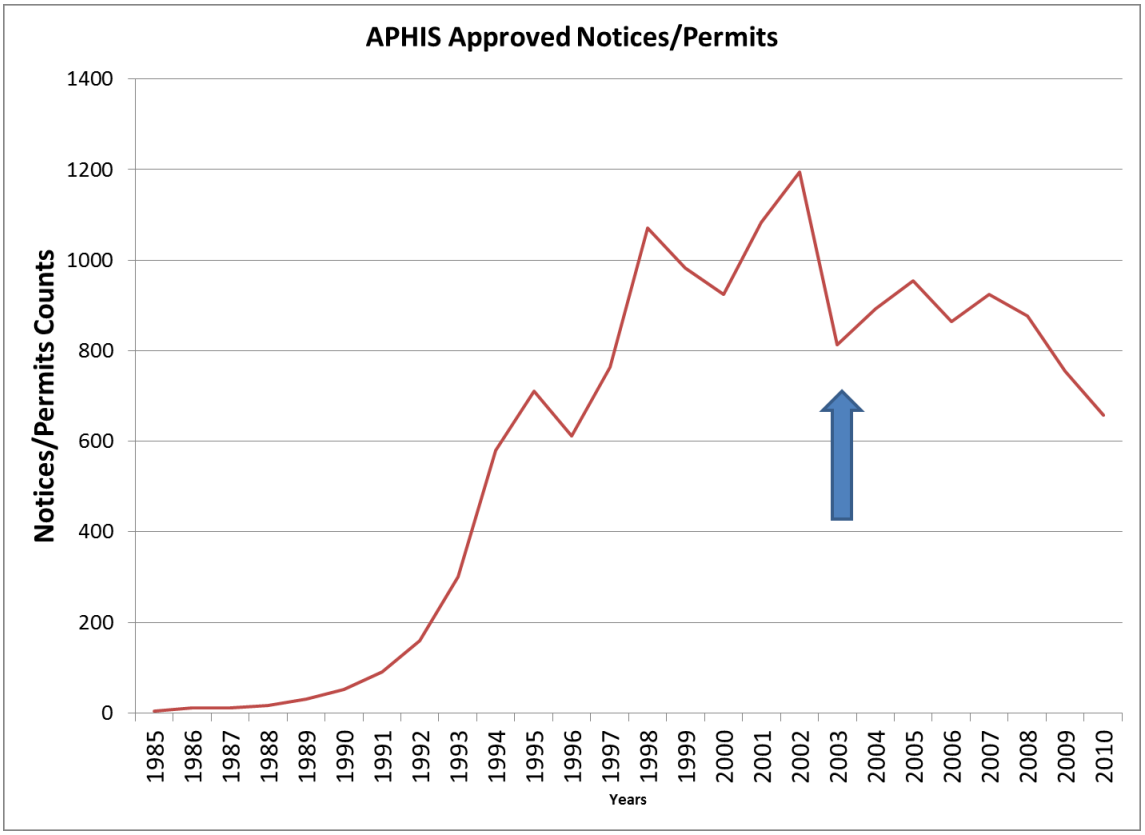

Fig. 1. Annual number of APHIS approved field tests through the notification and permit systems. The arrow denotes the beginning of ongoing litigation challenging the environmental impact of GE crops. Data from Information Systems for Biotechnology, 2011a.

Chapter 3

Genetically Engineered Kentucky Bluegrass: Blueprint for Circumventing Regulation

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In 2011, the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) announced that it lacked the authority to regulate Scotts Miracle-Gro Company's (Scotts) genetically engineered (GE) Kentucky bluegrass (*Poa pratensis* L.) variety (GE Kentucky bluegrass). As a consequence of this determination, Scotts is able to field test their new grass without government oversight or the limitations of confinement guidelines. Furthermore, Scotts may distribute and sell this product to U.S. consumers without going through APHIS's costly and time-consuming deregulation and environmental assessment process. This is an unexpected development and provides a blueprint for other biotechnologies to circumvent the regulation and environmental assessment of GE plants. In this article, we summarize relevant regulations and statutes, describe how Scotts was able to avoid regulation, and discuss other emerging technologies that are eluding regulation through piecemeal regulatory decisions. We conclude that the creation of a non-plant pest loophole is a consequence of APHIS's obsolete plant pest regulations, political gridlock between USDA's core constituencies, and a decade's worth of environmental litigation. A comprehensive overhaul of APHIS's regulatory system, or of the U.S. regulatory framework, is needed to assess the environmental risk of crop traits independent of the listed plant pests used in their creation.

Introduction

Many homeowners covet the perfect lawn—a dense green monoculture devoid of weeds. United States homeowners may be one step closer to achieving this goal. On July 1, 2011, during the contentious United States budget crisis debate, APHIS quietly issued an online press release declining to regulate Scotts GE Kentucky bluegrass (APHIS, 2011a). Like many other GE plants, GE Kentucky bluegrass has been engineered to be glyphosate-tolerant (APHIS, 2011a). Thus, homeowners may apply glyphosate to their GE Kentucky bluegrass turf and eradicate most or all weeds without damaging the grass itself. However, unlike previously regulated GE crops, GE Kentucky bluegrass can be field tested and commercialized without APHIS's oversight (APHIS, 2011a). This decision has ramifications far beyond the home lawn and is serving as precedent to guide emerging technologies on how to avoid regulation of genetically engineered crops. In order to understand APHIS's decision not to regulate GE KY bluegrass, we will review relevant regulations, the technologies taking advantage of this historic loophole, and the effect of environmental litigation on biotechnology.

Legal Background

In the United States, genetically engineered crops are subject to regulation by three agencies, depending on their characteristics: the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), and the USDA under the Coordinated Framework for the Regulation of Biotechnology (Office of Science and Technology Policy (OSTP), 1986). The FDA oversees the safety of human foods and

animal feed (OSTP, 1986) and the biotechnology industry may seek a voluntary consultation with the agency to confirm that the GE crop is "generally recognized as safe" (FDA, 2011). The EPA only regulates plant-incorporated protectants (PIPs), plants that are engineered to produce pesticides such as the *Bacillus thuringiensis* toxin for insect resistance or virus resistance (EPA, 2012). The USDA subdivision, APHIS, has promulgated regulations under the authority of the Plant Protection Act by which it oversees the transport, field testing, and commercialization of GE crops (APHIS, 2012a). Depending upon the GE plant's characteristics, the crop may be regulated by one or more of these federal agencies. However, particularly in light of some recent decisions by APHIS, some GE crops may not be regulated by any of these agencies. This article will focus on the paradigm-shifting regulation of GE crops by APHIS.

New GE crops must be field tested in accordance with APHIS's regulations. The vast majority of field tests proceed under the streamlined notification system (Information Systems for Biotechnology (ISB), 2012) wherein the field test applicant informs APHIS of its intent to conduct a field test, provides information such as the genetic engineering process, expected phenotype, location, and date of field testing. (APHIS, 2012a, 7 C.F.R. §340.3). Once APHIS determines that the crop is not a weedy species and does not cause disease, it "acknowledges" and thus approves the notification. The applicant must then agree to grow the crop in a confined and controlled field setting that prevents persistence of the crop or its offspring in the environment or commingling with non-GE crops (APHIS, 2012a). Not all crops can utilize APHIS's notification system. Perennials and biennials that require multiple years of continuous field testing (APHIS, 2008a) and crops engineered to produce biopharmaceuticals and industrial

products must undergo APHIS's more arduous permit system (APHIS, 2012a, 7 C.F.R. §340.4). This system requires an extremely detailed description of the molecular biology of the organism as well as customized safeguards to be implemented during the field test (APHIS, 2012a, 7 C.F.R. §340.4).

After a GE crop has been successfully field tested, the applicant may seek to deregulate the GE crop so that it may be sold and distributed like a conventional crop. The applicant must file a petition with APHIS for deregulation (APHIS, 2012a, 7 C.F.R. §340.6). Deregulation requires the applicant to demonstrate that the crop does not pose a plant pest risk. Information must be provided regarding the plant's taxonomy, relevant experimental data and publications, the plant's genotype and phenotype, the genetic engineering process, and the results of field tests (APHIS, 2012a, 7 C.F.R. §340.6). Furthermore, a detailed environmental assessment is required (National Environmental Policy Act, 2012) and thus, the deregulation process may take many years to complete. The additional threat of environmental litigation adds to the uncertainty and cost inherent in this process (McGinnis et al., 2012). Regulatory costs may add $6,000,000 to $15,000,000 (US$) in addition to crop development costs (Kalaitzandonakes et al., 2007), however the cost may rise further if the GE crop is mired in environmental litigation.

The question de rigeur is which GE crops may be captured under APHIS's regulatory authority and which crops may completely avoid the burden of regulation by using the non-plant pest loophole. Surprisingly, a fair number of new GE crops can legally escape field testing regulation and the costly deregulation petition process just like conventionally bred crops provided these GE crops avoid three regulatory triggers. The first trigger is APHIS's definition of a "regulated article" defined as any plant "which has

been altered or produced through genetic engineering" if any of the components including "the donor organism, recipient organism, or vector or vector agent belongs to" any of the USDA listed genera of plant pests and satisfies the definition of plant pest (APHIS, 2012a, 7 C.F.R. § 340.1). The definition of plant pest includes insects, bacteria, fungi, parasitic plants, viruses, and other organisms that cause disease or damage in plants. Listed plant pests include all *Agrobacterium* species and all viruses including figwort mosaic virus and cauliflower mosaic virus (APHIS, 2012a, 7 C.F.R. § 340.2), sources of strong constitutive promoters. A constitutive promoter is an unregulated promoter that allows continuous transcription of its associated gene or transgene. As defined, the use of *Agrobacterium*-mediated transformation or a viral promoter automatically triggers APHIS regulation. The second regulatory trigger is if the genetically engineered host plant or any of its genetic sequences come from organisms that are "unclassified". The term "unclassified" has not been defined in the regulations but will be briefly discussed in the next section. The third and catchall regulatory trigger is if the APHIS Administrator believes the GE organism to be a plant pest (APHIS, 2012a, 7 C.F.R. Sec. 340.1). This third trigger appears to imbue much regulatory discretion to the Administrator but has been largely ignored.

Scotts' GE Kentucky Bluegrass

On September 13, 2010, Scotts sent a letter of inquiry to the USDA seeking an official determination as to whether its GE Kentucky bluegrass would require APHIS's field testing and deregulation oversight (Scotts, 2010). This turfgrass is glyphosate-tolerant like many GE crops that were subject to field testing and the deregulation process

but with one important difference. No plant pests were used in the creation of this turfgrass and therefore, Scotts argued the turfgrass was not a "regulated article." The donor organism, recipient organism, vector, and vector agents were not plant pests. Kentucky bluegrass, the recipient organism, is not a listed plant pest, because it is not a parasitic plant (Scotts, 2010). The glyphosate tolerance gene, 5-enolpyruvylshikimate-3-phosphate synthase, came from the model plant *Arabidopsis thaliana* (L.) Heynh. (mouseear cress), arguably one of the world's most studied plants (Scotts, 2010). This is an innovation because previous generations of GE crops such as *Beta vulgaris* L. ssp. *vulgaris* var. *altissima* (sugar beet) derived glyphosate tolerance from the CP4 strain of *Agrobacterium tumefaciens* (APHIS, 2004), which is a listed plant pest. *Oryza sativa* L. (rice) was the source of both the ubiquitin promoter and the actin intron sequence used to produce the glyphosate-tolerance gene in this turfgrass. Previously, most promoters used in herbicide-tolerant gene constructs in other GE crops had been derived from viruses. Finally, the 3' untranslated region came from *Zea mays* L. (maize) (Scotts, 2010), and just like rice is not a plant pest. As for the vector, instead of using *Agrobacterium*-mediated transformation, Scotts utilized a gene gun to deliver the gene construct (Scotts, 2010). Thus, Scotts avoided the first plant pest trigger. In addition, Scotts argued that all of the donor organisms were "classified" or well-characterized because their genomes had been sequenced. Thus, this turfgrass did not implicate the second trigger.

In response to the letter of inquiry, APHIS concluded that GE Kentucky bluegrass is not a regulated article (APHIS, 2011b). Kentucky bluegrass and the donor organisms are not plant pests and are well classified so the first two regulatory triggers were not activated. The APHIS Administrator chose not to use his discretion to label the crop a

plant pest under the third trigger. Although APHIS concluded that it lacked the authority to regulate this turfgrass, it strongly encouraged Scotts to work with stakeholders to develop "appropriate and effective stewardship measures" (APHIS, 2011c). The relevant stakeholders and appropriate stewardship measures were not further defined.

The decision not to regulate GE Kentucky bluegrass was somewhat surprising in light of APHIS's litigation history with GE creeping bentgrass (*Agrostis stolonifera* L.). Genetically engineered Kentucky bluegrass was the subject of environmental litigation in conjunction with GE creeping bentgrass (*International Center for Technology Assessment (ICTA) v. Johanns*, 2007). The ICTA and its sister organization, Center for Food Safety (CFS), filed suit against Mike Johanns (former USDA Secretary) contesting APHIS's rejection of their 2002 petition to place GE creeping bentgrass and GE Kentucky bluegrass on the federal noxious weed list (*ICTA v. Johanns*, 2007). The ICTA and CFS also alleged in their suit that the USDA should have prepared an environmental assessment or impact statement before allowing field testing of GE creeping bentgrass, a wind-pollinated perennial, under the notification system. The second count of the lawsuit was more controversial, because GE creeping bentgrass escaped its regulated field test and a scientific study documented long distance pollen flow to plants 14 to 21 km distant from the field (Watrud et al., 2004). Genetically engineered creeping bentgrass was still being detected in eastern Oregon irrigation ditches as late as fall 2010 (Charles, 2011). While *ICTA v. Johanns* focused on gene flow risks such as the spread of the glyphosate-tolerance gene into sexually compatible *Agrostis* species, the case also discussed APHIS's authority under the Plant Protection Act (2000) to list or decline to list a plant as a federal noxious weed. The court concluded that APHIS wrongly denied ICTA's and

CFS's petition on the basis that APHIS used an impermissibly narrow definition of noxious weed derived from the superseded Noxious Weed Act (1974). Instead APHIS should have used the much broader noxious weed definition in the current Plant Protection Act (2000) that replaced the Noxious Weed Act (*ICTA v. Johanns,* 2007). In vacating APHIS's denial of the noxious weed petition and remanding it back to APHIS for further evaluation, the court opined that the Plant Protection Act (2000) still endows APHIS with much discretion to decide what species should be listed as a federal noxious weed (*ICTA v. Johanns*, 2007).

In conjunction with its 2011 determination that GE Kentucky bluegrass should not be regulated, APHIS also released its re-evaluation of ICTA's and CFS's 2002 petition to add GE Kentucky bluegrass to the federal noxious weed list (APHIS, 2011d). After considering the characteristics of both GE and non-GE Kentucky bluegrass, APHIS concluded that both types met the definition of a noxious weed under the Plant Protection Act because of their high potential to spread and become established particularly in grasslands and native prairies but not in agricultural production systems (APHIS, 2011e). Nevertheless, the USDA Secretary declined to list Kentucky bluegrass (GE and non-GE) as a noxious weed because the species posed considerably less of an impact potential (harm) than the 98 species that had been previously listed as noxious weeds (APHIS, 2011d). Interestingly, GE Kentucky bluegrass received nearly identical scores as the non-GE Kentucky bluegrass for both spread and impact potential.

It is likely that APHIS took into account that Kentucky bluegrass is one of the most prominent turfgrass species in the northern United States. Listing and eradicating one of the most popular home turfgrass species as a noxious weed would not only be

impractical, but would likely incur the ire of the American public. However, APHIS's documentation concluding that a GE grass engineered with an herbicide-tolerance gene carries approximately the same risk of invasiveness as its non-GE counterpart is somewhat scant considering the possibility of further litigation with ICTA and CFS. In its accompanying Weed Risk Assessment (APHIS, 2011e), APHIS did not discuss the likelihood that GE Kentucky bluegrass may hybridize with other *Poa* species and whether glyphosate-tolerance would enhance the weediness and impact potential of those species. Furthermore, APHIS failed to cite favorable scientific studies that supported its decision not to regulate Kentucky bluegrass. A prominent study showed that facultative apomixis in Kentucky bluegrass may significantly limit gene flow and hybridization (Johnson et al., 2006). However, apomixis is a double-edged sword that has not been thoroughly evaluated in GE plants. While this asexual form of reproduction may limit the spread of pollen containing the glyphosate-tolerance trait, facultative apomixis will ensure that the majority of the seeds from the transgenic mother plant will be clones carrying the herbicide-tolerance trait. Nevertheless, it is unlikely that another lawsuit would overturn the decision not to list GE Kentucky bluegrass as a noxious weed due to the broad discretion accorded to the USDA on this matter.

Biotechnologies Eluding Regulation: Letters of Inquiry.

Few plant biotechnologies escaped APHIS's regulation between 1990 and 2010. However, the developers of horticultural and agronomic crops with increasing frequency are sending letters of inquiry to APHIS asking for a determination as to whether their crops fall outside APHIS's regulatory authority. These letters of inquiry seeking to

exploit the non-plant pest loophole and APHIS's responses are quietly shaping national biotechnology policy as well as channeling investment in technologies that are perceived to have lower regulatory burdens. While APHIS's regulatory determinations have broad ramifications on emerging technologies, APHIS's response to these letters has largely escaped notice.

Cisgenics/Intragenics

Both cisgenesis and intragenesis involve the genetic engineering of a plant with DNA from a sexually compatible species. In cisgenesis, the gene must include its native introns, promoter and terminator, in the normal sense orientation (Schouten et al., 2006). Intragenic plants may contain DNA fragments from more than one sexually compatible species and the DNA fragments can be rearranged before they are integrated into the recipient plant's genome (Rommens et al., 2007). In theory, cisgenic and intragenic plants could have been bred conventionally.

New Zealand Crop and Food Limited set precedent for GE Kentucky bluegrass by seeking an opinion on the regulatory status of intragenic petunia (*Petunia* x *hybrida* hort. ex E. Vilm) genetically engineered for enhanced petal color (New Zealand Crop, 2007). The gene of interest, a MYB transcription factor that upregulates the anthocyanin pathway, and the chlorophyll A/B binding promoter were derived from petunia. The gene and its regulatory sequences were transferred via gene gun. The company also sought an opinion on a second construct that used the neomycin phosphotransferase (*NPTII*) gene from *Escherichia coli* as a selectable marker (New Zealand Crop, 2007). In its response, APHIS concluded that the first construct was intragenic and did not utilize DNA from plant pests; the second construct using a selectable marker was not intragenic, but *E. coli*

is not a listed plant pest (New Zealand Crop, 2007). More recently, APHIS exempted intragenic *Vitis* L. (grape) from regulation regardless of whether the plants were created by a gene gun or through protoplast engulfment of vector DNA (APHIS, 2012b).

Not all intragenic/cisgenic crops will escape APHIS regulation. Wageningen University and Research Center in the Netherlands recently inquired whether its cisgenic apple would fall within APHIS's regulatory purview (Wangeningen University, 2012). The University introduced an apple scab resistance gene (not identified but most likely Homologues of *Cladosporium fulvum* resistance genes *of Vf* region 2 (*HcrVf2*)*;* Joshi et al., 2011) with its native promoter and terminator into *Malus* x *domestica* 'Gala'. Despite the cisgenic origins of the genetic elements, APHIS refused to exempt this product from regulation, because Wageningen University researchers used *Agrobacterium*-mediated transformation instead of a gene gun (APHIS, 2012c). Therefore, these regulatory determinations show that most cisgenic/intragenic products will not be regulated by APHIS provided that no listed plant pest is used in the transformation.

Transgenic Grasses.

After the 2011 GE Kentucky bluegrass decision, APHIS assessed its authority to regulate GE switchgrass (*Panicum virgatum* L.), GE St. Augustinegrass (*Stenotaphrum secundatum* (Walter) Kunze), and a second form of GE Kentucky bluegrass (APHIS 2012d, e and f). In response to letters of inquiry, APHIS determined that these grasses did not fall under its regulatory authority, because the grasses did not utilize any plant pests in their creation. These regulatory determinations raise more questions than they answer. Ceres, Inc.'s switchgrass was genetically engineered to produce more biomass and more fermentable sugars (Ceres, 2012). While this trait is an advantage for ethanol production,

the unanswered question is whether this modification for increased biomass will make GE switchgrass invasive in native prairies. Conventional switchgrass is already considered a dominant member of the U.S. tallgrass prairie (Van Bruggen, 2003). Little is known about Scotts' second variety of GE Kentucky bluegrass except that it combines glyphosate-tolerance and "enhanced turfgrass quality" (Scotts, 2012a). Unfortunately, the gene for enhanced turfgrass quality was deliberately obscured (redacted) for confidentiality purposes. However, the gene most likely encodes gibberellic acid 2-oxidase, an enzyme that downregulates gibberellic acid production (Taiz and Zeiger, 2006). Scotts identified this gene from spinach (*Spinacia oleracea* L.) as the source of its enhanced turfgrass quality for St. Augustinegrass and APHIS did not redact this information (Scotts, 2012b). Metabolism of gibberellic acid may produce a shorter and greener phenotype (Scotts, 2012b). Reduction of gibberellic acid may reduce invasive potential, but this has not been evaluated. Genetically engineered switchgrass, St. Augustinegrass, and the second GE Kentucky bluegrass variety received even less scrutiny than the first GE Kentucky bluegrass variety, because APHIS was not required to conduct Weed Risk Assessments. There were no ongoing petitions to list these grasses as noxious weeds.

With respect to GE grass concerns, one of the most pressing questions is whether the adoption of glyphosate-tolerant turfgrasses will be a significant weed management risk. A regulated GE turfgrass would be subject to an environmental assessment or environmental impact statement prior to deregulation and such a document would most likely assess the cumulative impacts of increased glyphosate use (*Geertson Seed Farms v. Johanns*, 2007). Repetitive applications of glyphosate to agronomic crops without the use

of diverse herbicidal modes of action increase the selection pressure for glyphosate-resistant weeds (Duke and Powles, 2009). There is no reason to assume that selection pressure for herbicide-tolerant weeds will be less intense for a GE residential lawn than in an agronomic setting. Most turfgrasses are perennial so there is little likelihood of crop rotation that may require the use of different herbicides. Furthermore, homeowners that plant GE turfgrass may be drawn to the product because of the simplicity of applying one herbicide to eradicate weeds instead of using several weed specific pre-emergent and post-emergent herbicides. Unfortunately, APHIS cannot address these concerns, because it concluded that it lacks the authority to regulate these grasses.

Targeted Gene-Modification Technologies.

Kuzma and Kokotovich argued that targeted gene modification technologies would bring GE crop regulation to a "technical inflection point" (2011). On May 26, 2010, APHIS issued a response to Dow AgroScience, LLC's letter of inquiry regarding its use of zinc finger nuclease (ZFN) technology in maize (APHIS, 2010). The ZFN technology fuses a zinc finger DNA-binding domain to a nuclease to create a synthetic restriction enzyme that precisely induces a double stranded break in a specific sequence of DNA (Shukla et al., 2009). The technology can be used to insert chimeric genes, to create a mutation to knockout a gene, or to precisely alter one or more nucleotides (Weinthal, et al., 2010). In Dow's ZFN-12 corn, it produced a small deletion in the IPK1 gene, which encodes inositol-1,3,4,5,6-pentakisphosphate 2-kinase, an enzyme integral to phytate biosynthesis in maize seeds (Shukla et al., 2009; APHIS, 2010). This is a useful gene knockout because phytate in animal feed interferes with nutrient absorption. After examining the technology, APHIS concluded that no plant pests were used in the creation

of the ZFN-12 maize plants and therefore, it was not a regulated article (APHIS, 2010). However, techniques that are used to insert or alter genomic DNA will be considered on a case-by-case basis (APHIS, 2012g).

Another targeted gene-modification technology was evaluated by APHIS. Cellectis S.A. inquired whether its meganuclease platform was subject to APHIS regulation (Cellectis, 2011). Like the zinc finger nuclease, meganucleases cause a double strand break in a specific portion of the DNA. However, Cellectis collects naturally occurring restriction enzymes from organisms such as green algae (*Chlamydomonas reinhardii*) and then customizes the DNA sequences to match the region adjoining the area to be cleaved. Cellectis asked for a regulatory opinion regarding two types of alterations. The first type uses a cell's repair mechanism (non-homologous end joining) to close the break, create deletions, and results in a gene knockout. The second type repairs the break through homologous recombination using the introduction of a single nucleotide sequence or a specific single-stranded DNA sequence (Cellectis, 2011). With respect to the first type, APHIS opined that these GE plants would not be regulated articles as long as the host plant and the meganuclease are not from a plant pest (APHIS, 2011f). APHIS was more guarded with the second type of GE plants and said that determinations would be made on a case-by-case basis (APHIS, 2011f).

The decision by APHIS not to regulate targeted-gene modifications that use deletions to create gene-knockouts is not surprising. This type of modification is simply a more targeted means of mutagenesis (Kuzma and Kokotovich, 2011). Plants created by mutagenesis do not meet the definition of a regulated article. It is likely that APHIS will be forced in the near future to issue an opinion on its authority to regulate targeted gene-

modification techniques that use homologous recombination to either insert a gene or to alter a small number of nucleotides. Based upon precedent, APHIS will most likely state that the source of the inserted DNA will determine whether the final product is regulated.

Other Examples.

FasTrack breeding in *Nicotiana tabacum* L. (tobacco) at North Carolina State University was also evaluated by APHIS (APHIS, 2011g). This novel breeding system first introduces the *Flowering Locus T* (*FT*) gene from *Arabidopsis thaliana* under the control of the 35S Cauliflower Mosaic Virus constitutive promoter, and the *NPTII* selectable marker gene using *Agrobacterium*-mediated gene transfer (NC State, 2011). Using regulated transgenic methods, the *FT* gene results in early flowering, which facilitates an accelerated conventional plant breeding program because of a shorter juvenility period and faster generation times. The transgenic construct is removed after selecting for traits of interest through a backcross to a non-transgenic tobacco plant (NC State, 2011). Based upon this description, APHIS concluded that the final tobacco plants could not be distinguished from plants developed in a conventional breeding program and therefore could not be regulated (APHIS, 2011g). APHIS also issued a similar determination regarding FasTrack breeding in *Prunus* sp. L. (plum) (APHIS, 2011h).

The most recent use of null segregation involves the transformation of sorghum (*Sorghum bicolor* L.) with an RNAi transgene to down-regulate the gene *MUTS HOMOLOG 1* (*MSH1*; McKenzie, 2011). This process creates plants that display enhanced agronomic variation for several developmental phenotypes. Plants carrying the transgenic construct are crossed with wild-type sorghum and advantageous phenotypes are selected. After loss of the transgene through segregation, the epigenetic effect

continues (McKenzie, 2011). Recently APHIS confirmed that the final non-transgenic product would not be regulated, but the earlier progeny from the transgenic cross would be subject to regulation (APHIS, 2012h).

The preceding examples show that scientific innovation is alive and well. However, the relevant question becomes: does the prospect of lower regulatory burdens stimulate investment in new biotechnology or is new biotechnology simply outpacing the ability of federal agencies to revise their regulatory policies? We suspect that it is a little of both.

Conventional Crops with Transgenic Traits

Transgenic crops are being treated like conventional crops by APHIS. Conversely, unregulated conventional crops are bred with traits that were historically engineered into transgenic crops. For example, Kansas State University in conjunction with Dupont is developing herbicide-resistant *Sorghum bicolor* (L.) Moench (sorghum) through conventional means (Smith, 2012). Despite applying a cocktail of pre- and post-emergent herbicides, weedy grasses may not be sufficiently controlled in sorghum fields particularly in dry years. To remedy this problem, Kansas State developed two conventional varieties of sorghum that are tolerant of the acetolactate synthase (ALS) class of herbicides (Tesso, et al., 2011) and the acetyl Co-A carboxylase (ACCase) class of herbicides (Smith, 2012). Herbicide-resistance traits were identified in wild sorghum and crossed with crop sorghum varieties without using genetic engineering (Tesso et al., 2011). This is an interesting development because many critics have cited GE herbicide-resistant sorghum as a prime example of a crop that poses a very real threat of transgene

escape to weeds and serious economic and environmental injury. Consequently, a transgenic, herbicide-resistant sorghum has not been developed. Instead, we now have conventional sorghum lines with risks similar to that of a GE crop and APHIS lacks the authority to regulate this plant. A significant risk is that herbicide-resistant crop sorghum will hybridize with Johnsongrass (*Sorghum halepense* (L.) Pers.) and confer herbicide-tolerance to this sexually compatible weed (Arriola and Ellstrand, 1996). Johnsongrass has been ranked as one of the world's ten worst weeds (Holm et al., 1977), is strongly rhizomatous and can produce tens of thousands of seeds per plant. In addition to its invasive characteristics, Johnsongrass can produce high levels of cyanogenetic glycosides depending upon its growth stage that may be toxic to livestock (Howard, 2004). While herbicide-resistant sorghum may initially be used to control Johnsongrass, gene flow to the invasive weed is possible because sorghum's herbicide resistance gene is carried by wind-borne pollen. To make matters worse, sorghum can also hybridize with another closely related weed, shattercane (*Sorghum bicolor* subsp. *drummondii* Nees ex Steud de Wet & Harlan; Sahoo et al., 2010). Having sorghum varieties with two stacked forms of herbicide resistance can slow the development of resistant weeds. Nevertheless, farmers growing herbicide-resistant sorghum will need to implement best management practices such as eradicating sorghum relatives in nearby fields and being on the lookout for resistant weeds. Unfortunately, APHIS cannot ensure that best management practices are enforced.

The above letters of inquiry and the development of conventional herbicide-tolerant sorghum show the consequences of a plant pest-based as opposed to a trait-based regulatory system. The method of transformation such as the use of *Agrobacterium*, viral

promoters, or other plant pest sequences automatically triggers regulation. Meanwhile conventionally bred plants with the same traits and similar risks as GE plants avoid regulation. Some scientists and crop breeders are now deliberately avoiding the use of processes that trigger regulation. Consequently, APHIS may be rendered obsolete with respect to regulation of novel biotechnologies that carry a significant risk.

Why the Non-Plant Pest Loophole?

Why does the non-plant pest loophole exist and why is APHIS quietly opting out of regulating certain GE crops? Although APHIS has not published its rationale, there appears to be a suite of interrelated factors that created and enlarged this loophole. First, APHIS has not amended its definition of a regulated article since 1997. The regulated article definition with its single-minded focus on plant pests is at the heart of its regulatory scheme. This definition fails to consider that certain GE and non-GE crops may not be plant pests but rather pose a risk of creating a noxious or invasive weed. The 1997 revisions (APHIS, 1997) made minor changes but did not sufficiently anticipate that genetic engineering would move beyond *Agrobacterium*-mediated transformation and viral promoters. In the reality of 2012, APHIS is not keeping pace with rapidly evolving technologies and is becoming obsolete.

Secondly, APHIS as a subdivision of the USDA, lacked the political will to amend these regulations. In 2008, APHIS proposed a massive overhaul of its biotechnology regulatory system (APHIS, 2008b) to update the regulations to cover emerging technologies and to incorporate current scientific knowledge, but the proposed regulations did not gain support from the USDA's stakeholders. Herein lies the

challenge—the USDA represents stakeholders with different and adversarial interests. The USDA promotes and supports U.S. agriculture, but that includes industries, farmers, and consumers growing or consuming organic, conventional, and genetically engineered crops. Stakeholders are frequently at odds as indicated by a decade's worth of environmental litigation that entangled the USDA and APHIS (McGinnis et al., 2012). Consequently, the USDA and APHIS appear to be litigation weary and seeking to avoid regulating technologies that may instigate even more conflict and litigation.

The National Environmental Policy Act (NEPA, 2012) facilitated litigation by organic and environmental advocacy groups against APHIS regarding various GE crops. This environmental statute requires federal agencies including APHIS to evaluate whether "major Federal actions" will have a significant impact on the human environment (NEPA, 2012, 42 U.S.C. §4332). The statutory requirements add an additional layer of scrutiny to GE crops in addition to APHIS's regulations. Generally, APHIS's routine decisions to allow field tests of regulated GE crops are excluded from NEPA review but exceptions do exist for novel crops (McGinnis et al., 2012). All petitions to deregulate a GE crop qualify as major Federal action and either a concise environmental assessment or a more burdensome environmental impact statement must be prepared (APHIS, 2012i,7 C.F.R. §372.5(b)).

Since 2003, organic advocacy and environmental groups have challenged the sufficiency of APHIS's NEPA compliance in approving the field testing and deregulation of GE crops. These lawsuits alleged that APHIS failed to adequately evaluate and document the environmental impacts of biopharmaceuticals in food crops, glyphosate-tolerant creeping bentgrass, glyphosate-tolerant alfalfa (*Medicago sativa* L.), glyphosate-

tolerant sugar beet (*Beta vulgaris* L.), and cold-hardy eucalyptus (*Eucalyptus* hybrid) (McGinnis et al., 2012). After nearly a decade's worth of ongoing litigation, the opposition between APHIS's organic stakeholders and its GE crop stakeholders was entrenched. Consequently, this hostility made it extremely difficult for APHIS's stakeholders to reach a consensus as to how to prudently amend the regulatory system to take into account advances in science.

However, the non-plant pest loophole appears to shift the balance of power between APHIS and its diverse stakeholders. Previously, organic and environmental stakeholders used NEPA litigation to challenge the commercialization of GE crops. The number of field trials dramatically decreased after 2002 (ISB, 2012) as result of NEPA litigation (McGinnis et al., 2012). Furthermore, APHIS took substantially more time to deregulate GE crops (APHIS, 2012j). Now, the non-plant pest loophole appears to offer a path to significant lower regulatory costs and decreased NEPA litigation risk. Non-plant pest loophole crops appear to fall outside NEPA's statutory boundaries, because APHIS claims that it lacks the authority to regulate these crops. If APHIS is not engaged in a major federal action, then NEPA requirements for environmental review do not apply (*Alliance for Bio-Integrity v. Shalala*, 2000). Consequently, there may be no clear legal hook for an opposing group to file a non-frivolous NEPA lawsuit concerning crops that slip through the non-plant pest loophole. As a result, the non-plant pest loophole has become extremely attractive to both APHIS and biotechnology developers.

The non-plant pest loophole may give APHIS the leverage that it needs to get diverse stakeholders back to the bargaining table to reconsider massive revisions to the current regulations. Organic and environmental stakeholders that oppose GE crops are at

a crossroads as they watch their legal clout wane. Biotechnology developers are increasingly using innovative technology to completely avoid APHIS regulation. Organic and environmental stakeholders can no longer use NEPA litigation as a tool to delay and increase the costs of non-plant pest crops. Consequently, these stakeholders are faced with a choice: allow a new wave of biotechnologies to obviate regulation or else compromise. In direct contrast, biotechnology developers may be extremely reticent to amend the regulations to shrink the non-plant pest loophole. However, they might be coaxed back to the negotiating table if they can be assured of a more rational, streamlined regulatory system. Biotechnology developers would benefit from such a regulatory system because they can assure the public that their products are regulated.

Conclusion

Now is an auspicious time for APHIS and the USDA to overhaul the regulatory system to regulate based on crop traits and not on the of the plant pests used in their creation. This new regulatory system would not discriminate between conventional and GE crops which comports with the original intent of the Coordinated Framework of Biotechnology (OSTP, 1986). Instead, the focus would be on traits that pose a significant environmental or health risk regardless of their origin. We are not proposing a burdensome regulatory system that stymies scientific innovation. Instead, the regulations could reflect the 20+ years of experience that scientists have with GE crops and traits.

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Chapter 4

Environmental Control of Flowering in Pennsylvania Sedge

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Pennsylvania sedge (*Carex pensylvanica* Lam.) is an upland forest sedge with restoration and horticultural potential as a low maintenance groundcover for dry shade. For large landscape and restoration plantings, seed or achenes in this case, are much preferred due to lower labor and material costs. Pennsylvania sedge, however, typically produces few achenes in its native habitat. As a first step in improving achene production, this research evaluated the effect of vernalization and photoperiod on floral initiation and development. We conclude that Pennsylvania sedge is an obligate short day plant that does not require vernalization for flowering. Plants flowered when exposed to daylengths of 6 to 12 hours. Flowering was completely inhibited with 14-hour photoperiods. Pennsylvania sedge was florally determined after 4 weeks of 8-hour photoperiods. However, the largest number of normal inflorescences were produced with 6 to 10 weeks of 8-hour photoperiods. Inflorescence quantity varied by genotype.

Introduction

Pennsylvania sedge, a native of dry to mesic forests and savannas in the eastern half of Canada and the United States (Gleason and Cronquist, 1991), has both forest restoration and horticultural utility. This strongly rhizomatous species forms grass-like colonies (Curtis, 2006) that are suitable as a native groundcover (Meyer, 2004). Pennsylvania sedge is used for temperate forest restoration particularly in highly disturbed areas (Mottl et al., 2006). When planted densely in a managed landscape, it acts as a low maintenance groundcover that thrives in dry shade where grasses do not usually flourish (McGinnis and Meyer, 2011) and tolerates mowing (Darke, 2004). Thus, this attractive sedge fills a much needed niche for shade-loving, grass-like plants. For large landscaping projects, it would be advantageous to directly sow cold-stratified or after-ripened achenes (McGinnis and Meyer, 2011) instead of using vegetative propagules. Pennsylvania sedge normally flowers in April in the northern United States (Hipp, 2008) but typically produces few achenes in its native habitat (Curtis, 2006) although it is reported to be self-compatible (Friedman and Barrett, 2009). As a first step in improving achene production, the environmental factors influencing floral initiation and development were studied.

The ability to manipulate flowering is a first step in developing commercial seed production protocols. Temperature and photoperiod are two of the most important environmental factors in controlling flowering (Sung and Amasino, 2004). Vernalization is defined as the application of a cold treatment to a growing plant to promote flowering (Chouard, 1960; Taiz and Zeiger, 2006). By itself, vernalization does not result in floral

initiation, but rather removes an impediment to flowering (Gendall and Simpson, 2006). An extended exposure to a chilling treatment represses *FLOWERING LOCUS C*, a floral inhibitor gene in *Arabidopsis thaliana* (mouseear cress) winter annual phenotypes (Michaels and Amasino, 2000). The optimum vernalization temperature lies between 1 and 7 °C for a broad range of plants (Lang, 1965). Herbaceous plants in temperate zones are more likely to require vernalization such as spring flowering perennials *Euphorbia epithymoides* (cushion spurge), *Iberis sempervirens* 'Snowflake' (evergreen candytuft), and *Aquilegia* x *hybrida* (columbine) (Heins et al., 1997).

Many plants flower in response to a specific photoperiod (Garner and Allard, 1920). Photoperiods fall into five categories but the first three are the most prevalent categories: short day, long day, day neutral, intermediate day, and ambiphotoperiodic day plants (Thomas and Vince-Prue, 1997). Some plants require vernalization followed by long days to flower (Erwin, 2006).

Floral initiation and development in *Carex* spp. has been studied predominantly in northern European species. *Carex bicolor* (two color sedge) was determined to flower under long days while both *C. nigra* (smooth black sedge) and *C. canescens* (silvery sedge) required short days for floral initiation followed by long days for optimal floral development (Heide, 1997, 2002). In other species such as *C. echinata* (star sedge), *C. lachenalii* (twotipped sedge), *C. flava* (yellow sedge), *C. brunnescens* (brownish sedge), *C. atrata* (lesser blackscale sedge), *C. norvegica* (norway sedge), and *C. viridula* ssp. *viridula* (little green sedge), vernalization and short day photoperiods were interchangeable in promoting floral initiation. (Heide, 1997, 2002, 2004). The Arctic species, *C. bigelowii,* required a more complex sequence of short days for floral

initiation, a subsequent chilling treatment at 6 °C, and exposure to long days at 18 °C for optimum inflorescence heading and development (Heide, 1992). *Carex pensylvanica* is a member of *Carex* section *Acrocystis*, which is a section composed of sedges native to dry forests (Crins and Rettig, 2003). This is the first known report of flowering regulation in Acrocystis.

The objective of this research was to identify the environmental factors that regulate flowering in Pennsylvania sedge. We sought to determine whether this species requires vernalization, flowers in response to short or long day photoperiods, or requires a combination of vernalization and a specific photoperiod. In addition, we wanted to determine the critical photoperiod and the optimum number of weeks of inductive photoperiods for floral determination.

Materials and Methods

Vernalization and photoperiod requirements

On 2 July 2009, 20 plants were dug from a mixed seedling population (achene sources described in McGinnis and Meyer, 2011) maintained at the University of Minnesota Landscape Arboretum, Chaska, MN, USA. Plants were not flowering and no floral primordia were observed upon close inspection of the crown. The plants were divided into 80 uniform divisions with approximately 10 mature vegetative culms per division and the plants were randomized. Foliage was cut back to 6 cm and plants were transplanted into 475 ml square pots (8.89 cm diameter) containing Sunshine/Sungro SB500 High Porosity Bark Mix, (Seba Beach, AB, Canada). For 2 weeks, the plants were allowed to establish in a greenhouse with 22/18 °C day/night air temperature setpoints

and supplemental lighting from high-pressure sodium lamps was provided to extend the photoperiod to 16-h days. Plants were moved to four growth chambers (Environmental Growth Chambers, Chagrin Falls, OH, USA) for sequential vernalization and photoperiod treatments. Half of the plants were exposed to vernalizing temperatures of 5 °C (±2) for 12 weeks under either 16-h ("long day") or 8-h ("short day") photoperiods. The remainder of the plants were exposed to 12 weeks of either short day or long day photoperiods at 22 °C (±2) without a preceding vernalization treatment. After the initial 12-week treatment, plants were held for an additional 8 weeks under either short day or long day photoperiods at 22 °C. All combinations of treatments were conducted (Table 1). In all growth chambers, plants were subjected to combined fluorescent (Philips Lighting Co., Somerset, NJ, Model # F72T12/CW/VHO) and incandescent lights (Philips Lighting Co., Somerset, NJ, Model # 25A/IF) at irradiance levels of 200 umol·$m^{-2}s^{-1}$ at plant height and with a combined red to far red ratio of approximately 3:1. The relative humidity was 50%. All plants were watered as necessary and fertilized with Peters Professional Peat-Lite Special 20-10-20 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) at the rate of 200 ppm N every week throughout the experiment. Ten plants were randomly assigned to each of the eight treatment combinations. Two additional replications were conducted with start dates staggered at 2-week intervals. Percent flowering and days to first pollen shed were measured.

Critical photoperiod for floral initiation

Pennsylvania sedge cultivars were not available so achenes were collected and germinated in 2010 from the above-described mixed seedling population maintained at the University of Minnesota Landscape Arboretum. Four seedlings were selected based on vigor and vegetatively propagated to serve as individual genotypes. The genotypes were identified as MN101B, MN102O, MN103P and MN104R and were grown as stock plants under a 16-h photoperiod with temperature setpoints as described above.

Two weeks prior to the commencement of the experiment, stock plants were divided to produce uniformly sized plants with approximately 10 mature vegetative culms. Divisions were cut back to 5 cm and transplanted into 720 ml square pots (8.89 cm diameter) containing Sunshine/Sungro SB500 High Porosity Bark Mix. Plants from each of the four genotypes were exposed to one of five photoperiods: 6-, 8-, 10-, 12-, or 14-h photoperiods for 10 weeks in five growth chambers with both fluorescent and incandescent lights as described above. All plants were subjected to irradiance levels of 300 $\mu mol \cdot m^{-2}s^{-1}$, a temperature of 22 °C (±2), and 50% RH. A higher irradiance level was used in this experiment and the subsequent one in an attempt to speed floral initiation. The irrigation and fertilizer schedule were as outlined previously except that plants were irrigated with 300 ppm N of Peters Professional Peat-Lite Special 20-10-20. Two plants from each of the four genotypes were randomly assigned to each of the five photoperiod treatments for a total of eight plants per photoperiod treatment and 40 plants per replication. The experiment was replicated in time two additional times at 2-week intervals for a total of 120 plants. Data collected included days to visible bud, days to first

pollen shed, and number of inflorescences per plant. The term inflorescence refers to the stem bearing the terminal staminate spike with one or more subordinate pistillate spikes.

Minimum weeks of inductive treatments necessary for floral determination

The same four genotypes were divided and transplanted as described above. After allowing plants to establish in the greenhouse for 2 weeks under a 16-h photoperiod (temperature setpoints described above), all plants were placed in a growth chamber under an 8-h short day photoperiod for 0, 2, 4, 6, 8 or 10 weeks. Irradiance, temperature, relative humidity and fertilizer levels were consistent with the previous experiment. After exposure to 0, 2, 4, 6, 8, or 10 weeks of short day photoperiods, plants were transferred to a growth chamber programmed with a 16-h photoperiod (all other parameters being identical) for the remainder of the 10 weeks. After 10 weeks, all plants were returned to the greenhouse for an additional 4 weeks with a 16-h photoperiod. Three plants from each of the four genotypes were randomly assigned to each of the six treatments for a total of 12 plants per treatment. The experiment was replicated in time two additional times at 2-week intervals for a total of 216 plants. Data collected included days to visible bud, days to first pollen shed, and number of inflorescences at 14 weeks.

Statistical Analysis

Data were evaluated using Analysis of Variance (ANOVA: R Development Core Team, 2011). In all experiments, the percent flowering data was transformed using an arcsin, square root transformation because of the binomial distribution (Snedecor and Cochran, 1989). These data were then backtransformed and reported as percentages.

Multiple mean comparisons were conducted using Tukey's honestly significant difference test. Means were considered significant at the $P<0.05$ level.

Results and Discussion

Vernalization and photoperiod requirements

The effect of vernalization and photoperiod treatments on flowering percentages was significantly different ($P<0.001$) (Table 1). Twelve weeks of vernalization at 5 °C when followed by 8 weeks of either short day or long day photoperiods at 22 °C failed to produce a significant amount of flowering. Of the four vernalization treatments, flowering percentages ranged from 0% to 5.1% (Table 1). In direct contrast to vernalization treatments, non-vernalized (warm) plants that were exposed to short day conditions during the initial 12 weeks of the experiment flowered at the rate of 86% and 83% (Table 1). No plants flowered when exposed to 20 consecutive weeks of long day photoperiods (Table 1) regardless of vernalization.

Temperature and photoperiod treatments also had a significant effect on average days to first flower ($P<0.001$) (Table 1). For plants that were initially exposed to 12 weeks of short day photoperiods and warm temperatures, the average number of days to first flower was 92.7 and 94.6 d (Table 1.) The photoperiod following 12 weeks of short days did not significantly affect either flowering percentage or average days to first flower, because the plants were already florally determined during the initial 12 weeks. In contrast, the average number of days to first flower for vernalized plants that were subsequently exposed to short day conditions was 138 to 140 d (Table 1).

A very small percentage of vernalized plants (1.1% to 5.1%) flowered when exposed to short day photoperiods during the final 8 weeks (Table 1). Furthermore, no plants that were first subjected to long days and warm temperatures flowered when subsequently subjected to 8 weeks of short day conditions (Table 1). It is likely the vast majority of these plants would have flowered under short day conditions if the experiment had continued beyond 20 weeks. The failure of vernalization treatments to significantly promote flowering within the 20 week experiment was an unexpected result. Many early spring flowering herbaceous plants require or benefit from vernalization (Padhye et al., 2006). Vernalization allowed other *Carex* spp. such as brownish sedge, lesser black scale sedge, norway sedge, and little green sedge to flower under both long and short photoperiods (Heide, 1997). However, the shoot apical meristem in *C. pensylvanica* apparently is competent to produce floral primordia and does not require vernalization to remove an impediment to floral initiation.

Based upon these results, we conclude Pennsylvania sedge is an obligate short day plant that will not flower under continuous long day photoperiods. This photoperiodic requirement is similar to smooth black sedge and silvery sedge (Heide, 1997, 2002). In hindsight, it is not surprising that Pennsylvania sedge is not a long day plant based upon the timing of flowering in the northern United States. In the wild, the plant flowers in April in the northern United States (Hipp, 2008). It is one of the earliest woodland plants to flower in spring and has earned the nickname, early sedge. Pennsylvania sedge most likely is initiated and florally determined in the fall similar to *Carex lacustris* (hairy sedge) (Bernard, 1974) and then completes floral development in early spring when the snow melts.

Although Pennsylvania sedge was shown to be an obligate short day plant in this study, 14-17% of the plants failed to flower when exposed to 12 weeks of short day photoperiods, and we conclude that this is likely due to genotypic variation or poor vigor.

Critical photoperiod for floral initiation.

This experiment identified the critical photoperiod for flowering. Photoperiod produced a statistically significant effect on flowering percentages ($P<0.001$), but genotype did not. One hundred percent of each genotype exposed to 6-, 8-, 10-, and 12-h photoperiods flowered (Fig. 1). No plants flowered under the 14-h photoperiod (Fig. 1). Thus, the critical photoperiod is less than 14 h. Pennsylvania sedge is still considered a short day plant even though floral initiation took place with a 12-h photoperiod, because the plants only flower when exposed to fewer hours than the critical photoperiod (Thomas and Vince-Prue, 1977).

The main effects of photoperiod ($P<0.01$) and genotype ($P<0.001$) had a significant effect on average days to first flower. The genotype MN104R (6-h photoperiod) required on average 68 d to flower. In contrast, MN101B (8-, 10-, and 12-h photoperiods), MN102O (6-, 8-, and 10-h photoperiods), and MN103P (8-, 10-, and 12-h photoperiods) flowered on average in fewer than 60 d (Fig. 2). Within genotypes, there was no significant difference in average days to flower among photoperiods except a minor difference within genotype MN103P (Fig. 2). Overall, plants flowered considerably faster than the previous experiment because of increased fertilizer and irradiance levels as well as genotypic variation.

Photoperiod (P<0.001) and genotype (P<0.001) also affected the average number of inflorescences. When MN104R was exposed to 6-, 10-, and 12-h photoperiods, this genotype produced on average fewer than seven inflorescences per plant (Fig. 2). This was significantly less than the two highest flowering photoperiod treatments for MN101B (8-h and 12-h photoperiods) and the three highest for MN102O (8-h, 10-h, and 12-h photoperiods). The latter five treatment combinations produced in excess of 30 inflorescences per plant (Fig. 2). Within genotypes, there was no significant difference in the number of inflorescences produced across the range of photoperiods (Fig. 2).

Although not statistically significant, the 8-h photoperiod treatment produced the most consistent time to flower of all photoperiods. Average days to first flower under the 8-h photoperiod were less than 60 d for all four genotypes and had a range of 54.2 to 59.8 d (Fig. 2). Furthermore, MN101B, MN102O, and MN103P produced more than 25 inflorescences per plant and MN104R averaged 14 inflorescences under the 8-h photoperiod (Fig. 2). Based upon consistent results, the 8-h photoperiod was chosen as the standard photoperiod for the final experiment that sought to determine the minimum number of weeks required for floral determination.

Minimum weeks of inductive treatments necessary for floral determination.

The final experiment produced a clear distinction among treatments. The number of weeks of 8-h short day inductive photoperiodic treatments had a significant effect on the percent of plants flowering (P<0.001) while genotype had no significant effect. Plants exposed to 0 or 2 weeks of short day photoperiods failed to flower by the end of the 14-week experiment (Fig. 3). In contrast, 99% of plants flowered when exposed to 4 or 6

weeks of short days and 100% of plants flowered when exposed to 8 or 10 weeks of short days (Fig. 3). Number of weeks of short day photoperiods did not affect average days to first flower (data not shown). We conclude that Pennsylvania sedge plants are thus florally determined after exposure to 4 weeks of 8-h inductive treatments.

With respect to the average number of inflorescences produced per plant, an interaction occurred between the number of weeks of photoperiodic treatments and genotype (Table 2). Overall, MN104R produced fewer inflorescences than MN101B and MN102O when exposed to 6, 8, or 10 weeks of short day photoperiods (Table 2).

In this experiment, MN102O and MN103P produced abnormal inflorescences when they received fewer than 8 weeks of short day photoperiods. At just 4 weeks of short day photoperiods, MN103P regularly produced pseudoviviparous growth emanating from where the staminate and pistillate spikes should be (Fig. 4b). Pseudoviviparous growth is defined as the substitution of asexual propagules (plantlets) in place of sexual reproductive structures such as seeds (Elmqvist and Cox, 1996). When plants received 6 weeks of exposure to short days, pseudoviviparous growth replaced only the pistillate spikes in both MN102O and MN103P (Fig. 4c). While this is qualitative evidence, it does support exposing genotypes like MN103P and MN102O to a minimum of 8 weeks of short day photoperiods to ensure normal inflorescence development. In comparison, MN101B and MN104R showed an increase in male function when the plants received 10 weeks of short day photoperiods (Fig. 4d). Normally, the pistillate and staminate spikes are separate in this monoecious plant (Gleason and Cronquist, 1991). However, MN101B and MN104R regularly produced a tiny staminate spike on the tip of what should have been a purely pistillate spike when exposed to 10 weeks of 8-h photoperiods (Fig. 4d).

Therefore, determining the optimum number of weeks of inductive photoperiods for each genotype is imperative for producing a normal reproductive spike.

In all three experiments, all plants displayed a change in floral dichogamy or sequence. Pennsylvania sedge, a monoecious species, usually exhibits protogynous flowering that is defined as when the stigmas appear first and become receptive before the stamens reach anthesis (Friedman and Barrett, 2009). However, all Pennsylvania sedge plants in these experiments showed protandry; pollen shed preceded stigmatic presentation (data not shown). This observation can be beneficial in a commercial application if outcrossing is desired. If pollen shed completely precedes stigmatic presentation on an individual inflorescence culm, then pollination of the pistillate flowers using diverse pollen sources can be used to increase outcrossing rates in the inflorescence culms without emasculation. Pennsylvania sedge sets fewer seeds when it is self-pollinated versus artificially pollinated with pollen from other sources (Friedman and Barrett, 2009).

Conclusion

Based on this study, Pennsylvania sedge is an obligate short day plant and vernalization does not promote or accelerate flowering. Plants initiated flowers with photoperiods from 6 to 12 h. An 8-h photoperiod produced consistently high inflorescence production across four genotypes with an average of fewer than 60 d to first flower. All four genotypes used in this study required a minimum of 4 weeks of an 8-h photoperiod to be florally determined. However, depending upon genotype, 6 to 10 weeks of short day inductive conditions appear to be required for the production of

normal monoecious inflorescence culms. Based on the genotypic variation seen in this experiment, it is critical to select a genotype capable of producing a large number of normal inflorescences when attempting to propagate Pennsylvania sedge.

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Table 1. Temperature and photoperiod treatments affect flowering and average number of days to first flower in Pennsylvania sedge.

Treatment combinations	**Avg percent flowering (%)**	**Avg days to first flower**
Warm[z] LD-SD[y]	0.0 b[x]	NA[w]
Warm LD-LD	0.0 b	NA
Warm SD-SD	86 a	92.7 a
Warm SD-LD	83 a	94.6 a
Vern LD-SD	1.1 b	140 b
Vern LD-LD	0.0 b	NA
Vern SD-SD	5.1 b	138 b
Vern SD-LD	0.0 b	NA
P value	$P < 0.001$	$P < 0.001$

[z]During the first 12 weeks, plants were subjected to either warm (Warm) temperatures (22 °C) or vernalizing (Vern) temperatures (5 °C). All plants were exposed to warm temperatures during the final 8-week treatment.

[y]SD denotes short day photoperiods and LD denotes long day photoperiods. Plants were exposed to the first listed photoperiod during the initial 12-week treatment and plants were exposed to the second listed photoperiod during the final 8-week treatment

[x]Within column values followed by different letters are significantly different under Tukey's honestly significant difference test at $P \leq 0.05$.

[w]Plants marked with NA did not flower and therefore average days to first flower could not be calculated.

Table 2. Average number of inflorescences produced per plant by genotype and by weeks of exposure to 8-h photoperiodic inductive treatments.

Genotype	**4 Weeks**	**6 Weeks**	**8 Weeks**	**10 Weeks**
MN101B	29.7 efg[z]	53.7 bcd	71.7 ab	58.7 bc
MN102O	12.9 gh	46.2 cde	61.1 abc	78.9 a
MN103P	12.0 gh	37.0 de	35.8 de	58.9 bc
MN104R	9.38 h	15.1 fgh	33.3 ef	35.7 de
Significance				
Genotype x Weeks	0.00422			

[z] Average values within the table labeled with different lowercase letters were significantly different according to Tukey's honest significant difference test at $P \leq 0.05$.

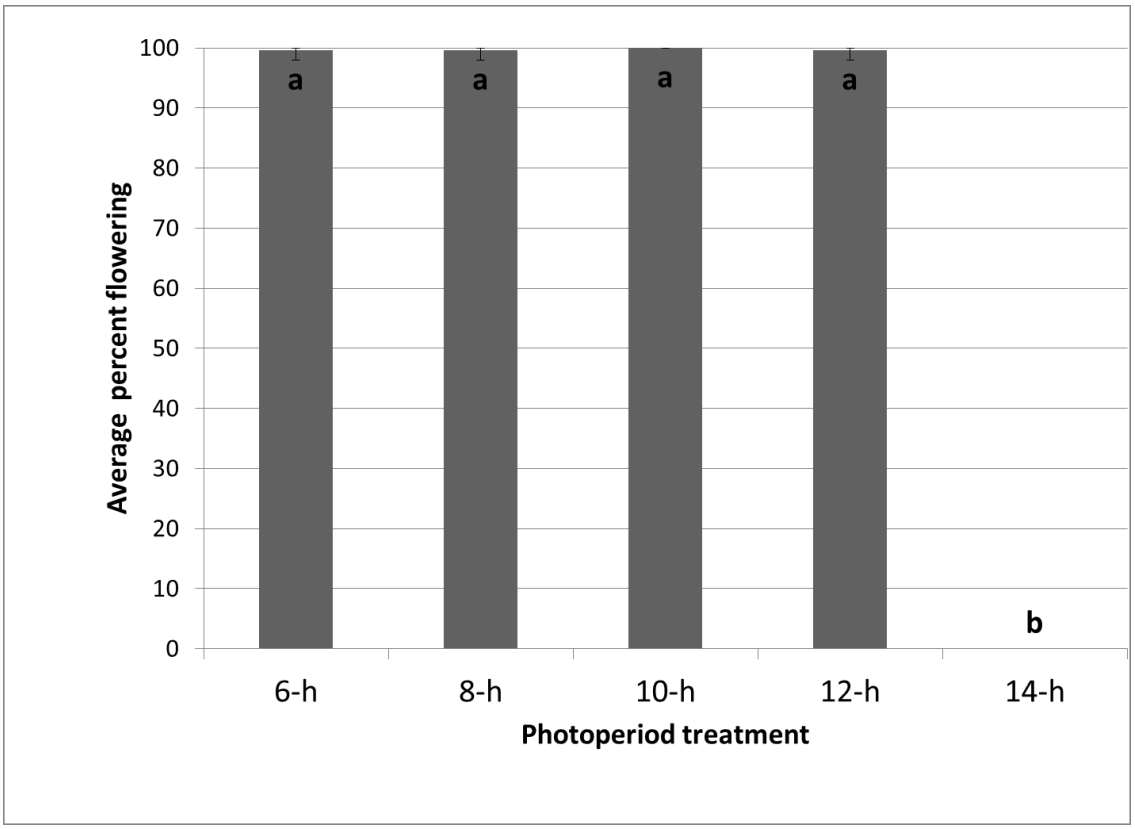

Fig. 1. Percent of Pennsylvania sedge plants flowering by photoperiod duration. Plants were subjected to 6-, 8-, 10-, 12-, or 14-h photoperiods. There were no significant genotypic differences. Bars labeled with different lowercase letters were significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. Standard error of the mean is indicated by error bars.

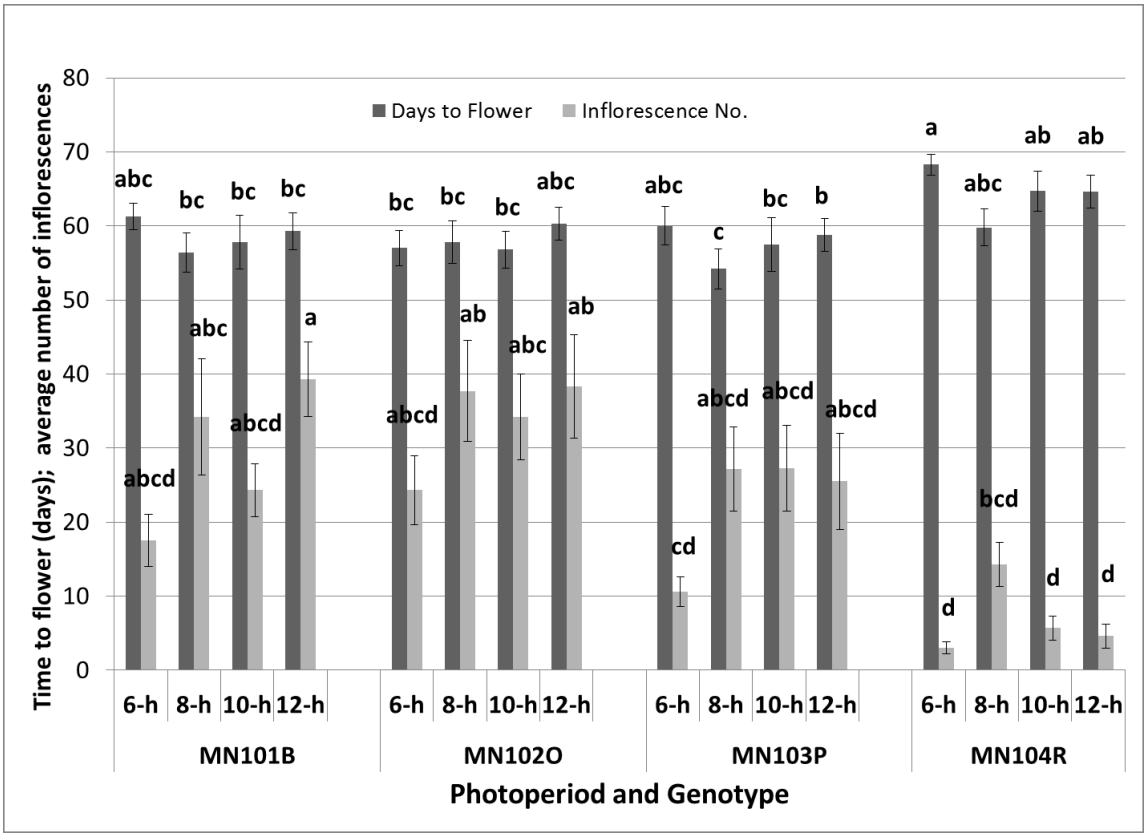

Fig. 2. Average number of days to first flower by photoperiod (in hours) and by genotype (MN101B, MN102O, MN103P, and MN104R) is depicted by dark gray columns. Average number of inflorescences by photoperiod and by genotype is depicted by light gray columns. Standard error of the means is indicated by error bars. Bars labeled with different letters were significantly different according to Tukey's honestly significant difference test at $P \leq 0.05$. Average number of days to flower was analyzed independently of average number of inflorescences.

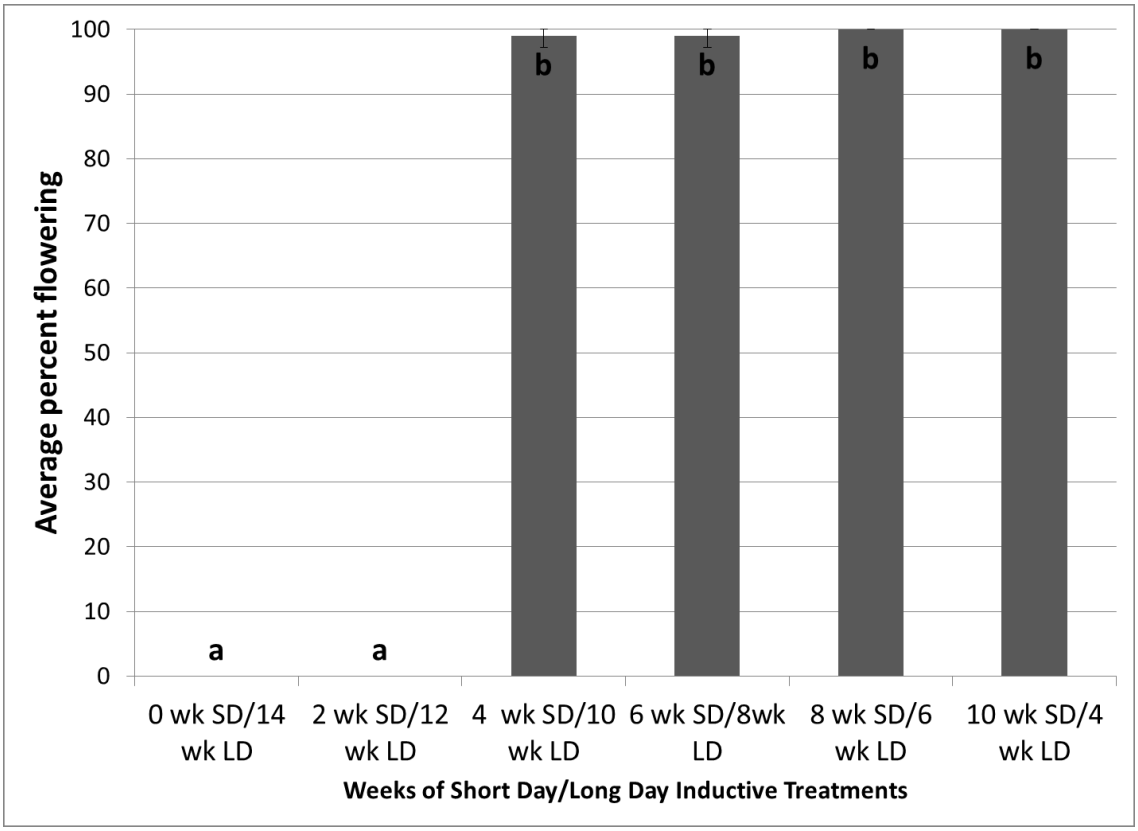

Fig. 3. Average percent of plants flowering when exposed to 0, 2, 4, 6, 8, or 10 weeks of short day (8-h) inductive treatments. Plants were then exposed to long days (16 h) for the remainder of the 14 week experiment. Data was pooled because genotypic differences were not significant. Bars labeled with different lowercase letters were significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. Standard error of the mean is indicated by error bars.

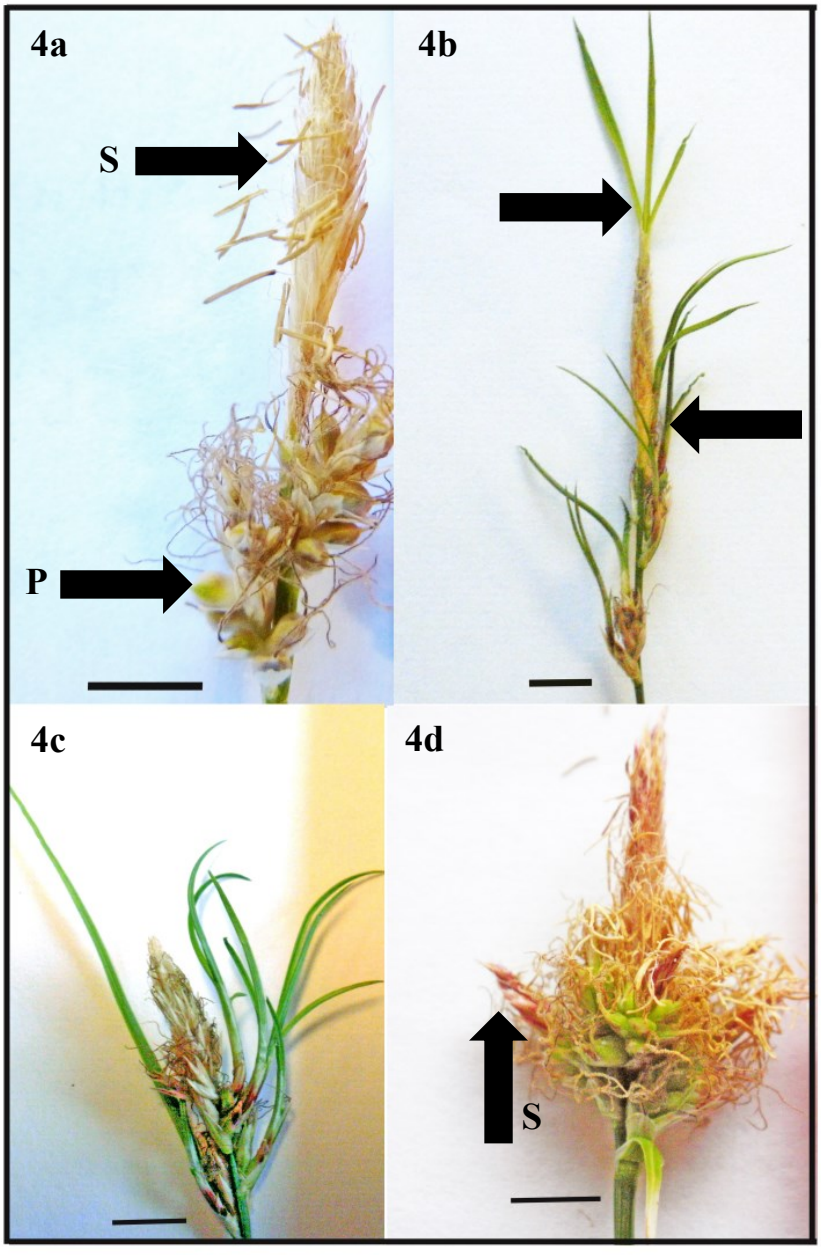
4a
S
P
4b
4c
4d
S

Fig. 4. Normal and abnormal inflorescences. Fig. 4a depicts a normal inflorescence with intact staminate spike (S) and subordinate pistillate spikes with perigynia (P), and dried stigmas. Note in Fig. 4b the pseudoviviparous growth that is originating from the terminal staminate spike and subordinate pistillate spikes in this plant that was exposed to 4 weeks of 8 h photoperiods. Fig. 4c shows the pseudoviviparous growth originating from the pistillate spikes in a plant that was exposed to 6 weeks of 8-h photoperiods. Fig. 4d illustrates an abnormal inflorescence from MN101B that has received 10 weeks of 8-h photoperiods. Note the staminate spikes (S) arising from what should purely be a pistillate spike.

Chapter 5

A post-induction chilling treatment controls dichogamy in *Carex pensylvanica*

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Abstract

Carex pensylvanica Lam. (Cyperaceae) is a monoecious, dichogamous forest sedge with temporal and spatial separation between male and female flowers. In its native habitat, *C. pensylvanica* is protogynous; the stigmas emerge and become receptive prior to anthesis. This study examined the environmental factors that control floral gender sequence and inflorescence culm heights in a series of four experiments. Plants were first subjected to three short day inductive photoperiods (8-, 10-, and 12-h) to evaluate how this impacted inflorescence height and dichogamy. The second experiment analyzed how different light spectra and temperature fluctuations affect floral stem height and dichogamy. The third experiment sought to determine the approximate date of floral bud initiation in the northern United States by digging field-grown plants at 2-week intervals during the fall and transferring them to a greenhouse under non-inductive photoperiods. The third and fourth experiments evaluated the effect of a chilling treatment on plants that were already induced. Photoperiod, light spectra, and temperature fluctuations failed to produce protogynous flowering. Plants were found to be determined and florally initiated in the fall in the northern United States. A post-floral induction chilling treatment (winter) was necessary to produce protogynous flowering and normal inflorescence culm elongation.

Introduction

Dichogamy, the temporal separation of male and female sexual function, is a relatively little studied phenomenon in both hermaphroditic flowers (intrafloral dichogamy) and in diclinous species (interfloral dichogamy) (Lloyd and Webb 1986). Protandry refers to the development of anthers and their dehiscence prior to stigma receptivity and frequently prior to stigma emergence; protogyny is the converse condition where stigmas precede anthers in floral development. Dichogamy may provide a complete temporal separation between male and female sexual functions (complete dichogamy) or there may be some overlap (incomplete dichogamy) (Lloyd and Webb 1986). Bertin and Newman (1993) examined 4,200 dichogamous species and concluded that dichogamy evolved to prevent interference between stamens and pistils and also to avoid self-fertilization. Recent studies of dichogamous species examined the degree of dichogamy (Cosmulescu et al. 2010; Griffin et al. 2000), synchronous dichogamy (Bhardwaj and Eckert 2001), whether early blooming female flowers are pollen limited in protogynous plants (Huang et al. 2002), and the effect of changes in environmental factors on the duration of stigma receptivity (Lora et al. 2011) and floral sex allocation (Bertin 2007). However, we could find no reports of environmental factors completely reversing the native sequence of flowering in a dichogamous, monoecious species. This project reports on the effect of environmental signals on floral gender sequence in *Carex pensylvanica*.

Carex pensylvanica (section *Acrocystis*) is a sedge native to dry woods with a broad distribution extending south from eastern Canada to Missouri and Georgia in the eastern United States (Ball and Reznicek 2002). Like most sedges, *C. pensylvanica* is a

wind-pollinated monoecious plant that produces separate male and female spikes on the same inflorescence culm (Hipp 2008). The inflorescence culm features a terminal staminate spike with one to three lateral pistillate spikes clustered near the base of the staminate spike (Gleason and Cronquist 1991). In floral development under native conditions, stigmas emerge and become receptive prior to anthesis (pollen shed), but there is a brief period in which both functions overlap (Friedman and Barrett 2009). *Carex pensylvanica* will set seed when it is self-pollinated but to a lesser degree than some North American forest sedges (Friedman and Barrett 2009). *Carex* is one of the world's largest angiosperm genera with over 2000 species (Reznicek 1990) mostly concentrated in the temperate latitudes of the Northern Hemisphere (Escudero et al. 2012).

Carex pensylvanica is a good model species to study environmental effects on dichogamy, because changes in flowering sequence have been observed in this species. During a previous flowering study, it was observed that plants grown in artificial environments usually display protandrous flowering while outdoor grown plants are protogynous (Chapter 4). In artificial environments, the terminal staminate flowers reach anthesis immediately after the staminate spike emerges from the leaf sheath surrounding the inflorescence. Thus, anthesis can occur before the subordinate pistillate spikes emerge from the leaf sheath (McGinnis, personal observation). This change in dichogamy was unexpected and provided the impetus for this study.

Based upon this reversal in floral gender sequence in artificial environments, two related hypotheses were formulated. The protogynous flowering sequence first requires inflorescence culm elongation in order for the subordinate pistillate spikes to emerge

from the leaf sheath and the stigmas to become receptive prior to the terminal staminate spike reaching anthesis. Second, environmental factors appear to control inflorescence growth and height and thus determine the sequence of male/female floral development in this species. These hypotheses were tested by posing three questions: (1) Do different photoperiods, light spectra, temperature, or a combination of these factors regulate inflorescence culm elongation and floral development? (2) At what time of year is *C. pensylvanica* flowering induced and initiated in Minnesota? (3) Does exposure to a chilling treatment after floral induction regulate inflorescence culm elongation and thus protogynous flowering?

Floral evocation, or the process by which a competent shoot meristem produces flowers instead of leaves, is comprised of three stages: floral induction, initiation, and development. Floral induction is the response to the developmental signal such as photoperiod that brings about a determined state (McDaniel et al. 1992). Determination is when the plant is committed to flower even after being removed from the appropriate development signals (McDaniel et al. 1992). Floral initiation is the production of flower buds. Floral development is the period of time after the formation of flower buds until anthesis (Erwin 2006). Floral development includes elongation of the inflorescence culm.

A recent study determined that *C. pensylvanica* is an obligate short day plant with a critical photoperiod of approximately 12 hours (h). Plants exposed to an 8-h photoperiod are determined to flower after 4 weeks at 22 °C although longer exposure enhances inflorescence number and flower development. Vernalization, a chilling treatment administered prior to floral induction to remove a physiological impediment to flowering (Taiz and Zeiger 2006), did not promote flowering in this species (Chapter 4).

No studies in *Carex* or other genera have reported a reversal in dichogamy based upon photoperiodic or other environmental signals. However, Heide (2004) reported that Norwegian populations of *C. flava* L. (section *Ceratocystis*) require separate photoperiods for floral induction and for floral development. *Carex flava* is determined under low temperatures and 10-h photoperiods, but subsequent long day photoperiods strongly promote inflorescence elongation and development. *Carex pensylvanica* plants that were induced under short day photoperiods and subsequently exposed to long day conditions did not exhibit protogynous flowering (Chapter 4).

This study first tested the effect of environmental factors including light quality and temperature on *C. pensylvanica* inflorescence culm height and flowering sequence. Lamps emitting different light spectra are known to influence internode length and plant height (Yorio et al. 1995). Metal halide lamps provide a spectrum of light energy that is more similar to natural daylight than fluorescent lamps (Shibuya et al. 2010), combined fluorescent and incandescent lighting (Duke et al. 1975), or red light-emitting diodes enriched with blue or far-red lighting (Brown et al. 1995). Although its spectrum is more natural, the effect of metal halide lamps on stem height is highly species (Duke et al. 1975) and cultivar (Yorio et al. 1995) specific. In a study of greenhouse grown agronomic crops, metal halide lamps increased plant height in *Medicago sativa* L. var. 'Saranac' (alfalfa) compared to combined fluorescent and incandescent lighting but no significant height differences were found in *Glycine max* L. var. 'Harosoy' (soybean), Zea mays L. var. 'NK Pk-446' (corn), or *Phleum pretense* L. var. 'Climax' (timothy grass) (Duke et al. 1975).

Temperature may also regulate inflorescence and plant height. Erwin et al. (1989) developed a technique to manage plant height by controlling diurnal temperature fluctuations. When day temperature exceeds night temperature, stem elongation occurs at a greater rate than at a constant temperature or conversely when the night temperature exceeds day temperature (Erwin et al. 1989). This concept is known as DIF and the response is quantitative--the greater the difference between day and night temperature, the greater the stem elongation (Erwin et al. 1989). While greenhouse growers usually limit DIF to prevent excessive elongation, this study maximized DIF to increase inflorescence height in *Carex pensylvanica*.

Knowledge of the life cycle of *C. pensylvanica* would be useful in understanding the timing of environmental signals that plants are exposed to outdoors and how these environmental signals affect floral induction, initiation, development, and dichogamy. *Carex pensylvanica* flowers in April and the achenes form in May and June in the northern United States (Hipp 2008). However, little is known about when floral induction and initiation occurs in *C. pensylvanica* or in other species. Nine *Carex* species were shown to be induced and initiated by July or August and to go dormant in November when grown in a greenhouse in the United Kingdom (Smith 1966). *Carex lacustris* Willd. forms flower buds in fall in the northern United States, the inflorescence grows to 1.0 cm by November, and then the plant flowers in late spring (Bernard 1975). The seasonal timing of floral initiation is important, because the production of flower buds in autumn may signal a need for a subsequent chilling treatment for optimal inflorescence culm elongation, floral development, and protogynous flowering. This proposed chilling treatment should not be confused with a dormancy-breaking treatment. A dormancy-

breaking treatment is the application of a cold treatment after floral induction to overcome arrested floral development (Erwin 2006). *Carex pensylvanica* plants do not exhibit arrested floral development (Chapter 4). The plants will flower in the absence of a dormancy-breaking treatment; albeit with reversed gender sequences. Most *Carex* species do not require a post-induction chilling treatment to flower normally (Heide 1997, 2002, 2004). The only *Carex* species that benefits from a chilling treatment applied after floral induction is *Carex bigelowii* Torr. ex Schwein., an Arctic sedge that exhibited accelerated floral development after a 6-week chilling treatment (Heide 1992). However, the chilling treatment failed to significantly influence inflorescence culm height in this Arctic species (Heide 1992).

Materials and Methods.

Plant Materials for Experiments 1, 2, and 4.

Two clonal genotypes were used for Experiments 1, 2, and 4. Short day selections, MN101B and MN102O, are induced under photoperiods ranging from 6 to 12 h at 22 °C. When exposed to 8-h photoperiods, both genotypes are committed to flowering after 4 weeks; however longer exposure is required to produce the maximum number of inflorescences (Chapter 4). MN102O requires 8 to 10 weeks of 8-h photoperiods for optimal inflorescence production while MN101B only requires 6 weeks of exposure (Chapter 4).

Stock plants for both genotypes were maintained in a greenhouse (maximum and minimum temperature setpoints of 21 and 18 °C) under a 16-h non-inductive photoperiod. Supplemental lighting from metal halide bulbs (Philips Lighting Co.,

MH400/U, model no. 34415-0, Somerset, NJ, USA) was used to extend the day length. Two weeks prior to each experiment's commencement, stock plants were divided to produce divisions with approximately 10 vegetative culms. The clonal divisions were planted into 720 ml square pots (8.89 cm) diameter containing Sun Gro Horticulture Metro-Mix 950 (Vancouver, BC, Canada), a high porosity growing medium. The plants were allowed to establish in the greenhouse for 2 weeks before being randomly assigned to experimental treatments in growth chambers. Throughout all experiments, plants were fertilized weekly with of Peters Professional Peatlite Special 20-10-20 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) at the rate of 300 ppm N. Plant materials for Experiment 3 will be discussed in a subsequent section.

Experiment 1: Influence of photoperiod on inflorescence height.

Plants were randomly assigned to three growth chambers (Environmental Growth Chambers, Chagrin Falls, OH, USA). Each growth chamber was programmed to provide an 8-h photoperiod using both fluorescent (Philips Lighting Co., Somerset, NJ, USA, Model #F72T12/CW/VHO) and incandescent bulbs (Philips Lighting Co., Somerset, NJ, USA, Model #25A/IF). For the initial 8 h, the irradiance level was 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant height and the combined red to far-red ratio (R/FR) was approximately 3:1. The control treatment received just the 8-h photoperiod (8-h) without day extension lighting. The second and third treatments received either an additional 2-h or a 4-h incandescent day extension for combined photoperiods of 10 or 12 h (irradiance level 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and R/FR: 0.64). Incandescent lighting was used to prevent confounding the experiment by significantly increasing the daily light integral. For all treatments, the relative

humidity was approximately 50% and the temperature was 22 °C (±2) at plant height. Plants were maintained in growth chambers for 10 weeks. Three plants from each of the two genotypes described above were assigned to each of the three treatments. Two replications in total were conducted with start dates spaced 2 weeks apart. Data collected included percent of plants flowering, inflorescence height at anthesis, inflorescence height at stigma appearance, days to anthesis, days to stigma appearance, and number of inflorescence culms per plant. Height at anthesis and at stigma appearance (in cm) was measured from the level of the soilless media to the tip of the staminate spike. Data were collected on the first two inflorescences for each plant and the values were averaged.

Experiment 2: Influence of spectral quality and diurnal temperature fluctuations on inflorescence height.

Four growth chambers were programmed to provide an 8-h inductive photoperiod, irradiance levels of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and approximately 50% relative humidity. The first two growth chambers were equipped with fluorescent and incandescent light bulbs (described in the previous experiment) that provided R/FR of approximately 3:1. The second two growth chambers contained metal halide bulbs (Philips Lighting Co., MH400/U, model no. 34415-0, Somerset, NJ, USA) with an R/FR of approximately 4.7:1. Of each pair of growth chambers, one was programmed at a constant 22 °C (±2) and the other at diurnally fluctuating temperatures of 22 (day)/12 (night) °C (±2) with 2 h ramping transitions in between the two temperatures for an average temperature of 17 °C. The experiment continued for 10 weeks. Three plants from each genotype were assigned to each of the four treatments. Three replications in total

were conducted with start dates staggered at 2-week intervals. Data collected was the same as for the previous experiment.

Experiment 3: Fall Initiation Experiment.

This experiment sought to determine whether *C. pensylvanica* plants are determined and initiated to flower in the fall in Minnesota, USA (latitude 45°, USDA plant hardiness zone 4) and whether a subsequent chilling treatment would ensure normal inflorescence elongation and protogyny. For this experiment, mature plants were dug in 2011 from a *C. pensylvanica* population of field grown plants (achene sources described in McGinnis and Meyer 2011) maintained at the University of Minnesota Landscape Arboretum, Chaska, MN, USA. Prior to removal, the plants were grown in full sun and received periodic summer irrigation but were not fertilized. None of the plants were visibly flowering when they were removed from the field. Plants were removed from the field on the following dates (daylength in parentheses): 22 September 2011 (12 h, 14 min), 6 October 2011 (11 hr, 27 min), 20 October 2011 (10 h, 45 min), and 4 November 2011 (10 hr, 2 min).

After the plants were uniformly divided and transplanted in the same manner as previous experiments, they were randomized between two treatments: (1) Ten plants (unchilled plants) were placed in a greenhouse (maximum and minimum temperature setpoints of 21 and 18 °C) with supplemental metal halide lighting to extend the photoperiod to 16-h noninductive long days; (2) Ten plants were exposed to a chilling treatment (chilled plants) in a growth chamber for 10 weeks at 5 °C, approximately 50% relative humidity, a 16-h photoperiod, and irradiance levels at 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After 10

weeks, the chilled plants were moved to the same greenhouse as the unchilled plants. The treatments were repeated for each of the four removal dates. In total, 80 plants were used for this experiment.

Data were taken on the first six inflorescence culms for each plant and averaged. Data were collected per inflorescence culm as described above. In addition, average days to first flower was calculated. For the unchilled plants, the days to first flower were simply the number of days between the date the plants were placed in the greenhouse and the first flower (male or female). For the chilled plants, the days to flower excluded the 10-week chilling treatment and only counted the days after plants were moved to the greenhouse. This experiment was not replicated, but results from it were used to design the last experiment. In addition to the above experiment, field-grown plants at the University of Minnesota Landscape Arboretum were visually inspected on 18 November 2011 and assessed for inflorescence production.

Experiment 4: The effect of a post-induction chilling treatment on dichogamy.

In this experiment, *C. pensylvanica* genotypes MN101B and MN102O were exposed to three sets of sequential temperature treatments to evaluate the effect of a post-induction chilling treatment on inflorescence height and dichogamy. For the purposes of this paper, a post-induction chilling treatment (chilling treatment) is the application of cold temperatures to a plant that has been induced under short day photoperiods for 6 weeks.

In Treatment 1, all plants were exposed to irradiance levels of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$, approximately 50% R.H., and 8-h photoperiods to induce flowering. The variable in

Treatment 1 was temperature. Half of the plants were exposed to temperatures that mimicked the average high and low temperatures for September in Minneapolis/St. Paul, MN, USA (latitude 45°) (Minnesota Climatology Working Group 2012). These plants were exposed to day temperatures of 18 °C (±2) for 10 h and night temperatures of 7 °C (±2) for 10 h with 2 h of transitional ramping to reach the desired temperatures. The average daily temperature was 12.5 °C and this treatment combination was referred to as COLD. The remaining half of Treatment 1 plants were exposed to a constant temperature of 18 °C (±2) (WARM). Treatment 1 was conducted for 6 weeks, after which plants were moved into Treatment 2.

Half of the plants in Treatment 2 were exposed to a post-induction chilling treatment for 10 weeks at 4 °C (±2) in a cooler (The Vollrath Company, Sheboygan, WI, USA). Overhead lighting was provided by a metal halide bulb for 8 h and irradiance levels were approximately 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The remaining plants in Treatment 2 did not receive a chilling treatment but were instead moved directly into Treatment 3. For Treatment 3, plants were moved to growth chambers with a 16-h noninductive photoperiod. Half of Treatment 3 plants were subjected to temperatures that mimicked the daily high and low temperatures during *C. pensylvanica* spring floral development in Minnesota (latitude 45°) (Minnesota Climatology Working Group 2012) namely fluctuating temperatures of 17 °C (±2) for the duration of the 16-h day and 5 °C (±2) during the 8-h night. The daily average temperature was 13 °C (COLD). The remaining plants in Treatment 3 were exposed to a constant 17 °C (±2) temperature (WARM).

All eight combinations of treatments were tested. Three plants from each of two genotypes, MN101B and MN102O, were placed in each treatment combination for a total

of 24 plants per replication per genotype. For MN101B, two replications in total were conducted due to scarcity of stock plants. For MN102O, three replications were conducted. Data was taken on the first six inflorescence culms for each plant and the values were averaged. Data was collected as previously described.

Statistical Analysis

Average height at anthesis, average height at stigma appearance, average difference in height between stigma appearance and anthesis, and average difference in days between stigma appearance and anthesis for each experiment were separately evaluated using analysis of variance (R Development Core Team 2012). Average difference in height was calculated by subtracting average height at stigma appearance from average height at anthesis. A negative value for average difference in height shows protandrous flowering (male function first) and a positive value shows protogynous flowering (female function first). Average difference in days between stigma appearance and anthesis was calculated by subtracting average days to stigma appearance from average days to anthesis. Likewise a negative value shows protandry and a positive value shows protogyny. The two values were important because they quantify the flowering sequence. Multiple mean separations were conducted using Tukey's HSD test. Means were considered significant at the $P<0.05$ level.

Results

Experiment 1: Influence of photoperiod on inflorescence height.

All genotypes responded with protandrous flowering under all treatment conditions, which was the reverse sequence of normal protogynous floral development as previously described in native populations of *Carex pensylvanica* (Fig. 1). Average inflorescence height at anthesis ranged from 3.7 to 5.8 cm while average height at stigma appearance ranged from 7.1 to 8.8 cm (Fig. 1). With respect to average height at anthesis, statistically significant differences occurred between treatments but were not sufficient to produce protogyny ($P \leq 0.05$). There were no statistically significant differences in average height at stigma appearance, average difference in height, or average difference in flowering dates. Male function preceded female function by 5.4 to 9.6 days (Fig. 1).

Experiment 2: Influence of spectral quality and diurnal temperature fluctuations on inflorescence height.

All treatment combinations produced abnormal protandrous flowering; neither genotype nor lighting source had a significant effect on height or timing of staminate or pistillate flowering ($P \geq 0.05$). However, diurnal temperature fluctuations yielded significantly taller culms at anthesis (6.5 cm) compared to a warmer, constant temperature (5.2 cm) (Fig. 2) but not for average height at stigma appearance. Anthesis preceded stigma appearance on average by 3.5 days under fluctuating temperatures compared to 6.7 days for constant temperatures.

Experiment 3: Fall Initiation Experiment.

Plants dug on 22 September 2011, 6 October 2011, 20 October 2011, and 4 November 2011 and directly placed in a greenhouse (unchilled plants) under a noninductive 16-h photoperiod flowered at rates of 40, 70, 100 and 90%, respectively (Table 1). Chilled plants flowered at the rate of 90 to 100% regardless of date removed from the field (Table 1). Mean separation was not conducted on the average flowering percent due to the lack of replication. The average time to first flower for unchilled plants greatly decreased from 41 days (plants dug on 22 September 2011) to 17 days (plants dug on 4 November 2011) ($P \leq 0.05$). In comparison, average days to first flower for chilled plants was remarkably consistent for all dates and ranged from 13 to 15 days after the completion of the chilling treatment (Table 1).

Unchilled plants that were dug on 22 September 2011 and 6 October 2011 displayed protogynous flowering while unchilled plants dug on 20 October 2011 and 4 November 2011 displayed protandrous flowering. Plants dug on the first two dates did not reach anthesis until the average inflorescence height was 12.4 and 14.7 cm respectively. Conversely, plants dug on the last two dates were considerably shorter and reached anthesis at the average height of 7.6 and 6.4 cm (Table 1).

In direct contrast to unchilled plants, all plants that received a chilling treatment displayed protogynous flowering regardless of date they were removed from the field. Average height at anthesis ranged from 13.5 to 15.2 cm while average height at stigma appearance ranged from 8.9 to 12.5 cm (Table 1). Furthermore, female function preceded male function by 1.3 to 5.3 days for all four dates (Table 1).

Inspection of plant crowns growing outdoors on 18 November 2011 revealed visible signs of floral initiation and development. Floral buds were found to be visible to the naked eye and had grown to over 2 cm in length (Fig.3) supporting the conclusion that floral initiation does take place in fall.

Experiment 4: The effect of a post-induction chilling treatment on dichogamy.

In this fourth experiment, *C. pensylvanica* plants were exposed to three sequential temperature treatments to: (1) induce the plants, (2) provide a post-induction chilling treatment, and (3) promote normal inflorescence development. The two genotypes showed different responses to the combination of sequential temperature treatments. MN101B responded to the chilling treatment with protogynous flowering. Regardless of the temperature during floral induction (Treatment 1) or during floral development (Treatment 3), MN101B plants that received a 10-week chilling treatment flowered in a protogynous sequence. The chilled inflorescence culms first reached stigma appearance at an average height of 12.7 to 15.2 cm and then anthesis at 16.1 to 21.9 cm (Fig. 4). In contrast, unchilled plants from MN101B were protandrous and reached anthesis first at an average height of 7.6 to 11.6 and subsequently stigma presentation at 8.6 to 12.0 cm (Fig. 4). Average difference in flowering dates between stigma appearance and anthesis for plants receiving a chilling treatment showed that female function preceded male function on average by 2.7 to 4.9 days (Fig. 4). For unchilled plants, male preceded female function by an average of 1.6 to 6.3 days (Fig. 4).

Results from the chilling treatment for genotype MN102O were less distinct than those for MN101B. While chilled MN102O plants did not reach anthesis until they

attained a height of 15 cm or greater, this was also true for one other unchilled treatment combination (Fig. 5). Only two treatment combinations displayed protogynous flowering: (1) chilled plants that were exposed to cold temperatures during Treatments 1 and 3 and (2) unchilled plants that were exposed to cold temperatures during Treatments 1 and 3. The rest of the treatment combinations exhibited protandrous flowering or synchronous flowering (Fig. 5). Genotype MN102O further differed from MN101B in that abnormal flowering was observed in all treatment combinations. Normally, the terminal spike is unisexual and composed solely of staminate flowers (Gleason and Cronquist 1991). However, stigmas intermingled with the stamens on the terminal spike in MN102O (Fig. 6).

Discussion

Manipulation of photoperiod, spectral quality, and diurnal temperature fluctuations did not produce protogynous flowering.

Using only 8-, 10-, and 12-h photoperiods, the inflorescence culms barely emerged from the leaf sheath and anthesis occurred before the culm reached 6 cm (Fig. 1). In most cases, this was before the subordinate pistillate spikes emerged from the leaf sheath. Thus, increasing the photoperiod from 8 to 12 h failed to produce normal protogynous flowering (Fig. 1). More substantial growth was expected, because photoperiodic day extension was provided solely by incandescent bulbs with a low red to far red ratio of 0.64. End of day lighting that is rich in far red mimics twilight and can result in significant stem elongation (Blom et al. 1995). However, manipulation of

photoperiod and end of day lighting failed to produce stem elongation or protogynous flowering in *C. pensylvanica*.

Metal halide lighting, as compared to combined fluorescent and incandescent lighting, did not significantly affect inflorescence culm height or flowering time. This result was expected, because the effect of metal halide lighting on stem elongation is highly species (Duke et al. 1975) and cultivar (Yorio et al. 1995) specific. However, large fluctuations between day and night temperatures caused a small increase in inflorescence culm elongation at anthesis compared to constant temperatures (Fig. 2). While diurnal temperature fluctuations affected flowering height and time, it did not induce normal protogynous flowering.

Carex pensylvanica plants are initiated in the fall.

The vast majority of unchilled plants dug on 6 October 2011, 20 October 2011, and 4 November, 2011 flowered after being placed in a greenhouse without additional photoperiodic treatments or chilling to enforce induction and development. Furthermore, 2 cm long floral buds were found on 18 November, 2011 after a visual inspection of outdoor grown plants. These results show that *C. pensylvanica* plants are induced and initiated by late fall in the northern United States. The plants then complete floral development and bloom with the first few warm days of spring, similar to other reports (Hipp 2008) and in Minnesota (McGinnis, personal observation). *Carex lacustris*, a North American wetland sedge, exhibited a very similar life cycle and the inflorescence grew to 1.0 cm by November (Bernard 1975).

Plants from all four dates that received a 10-week chilling treatment displayed normal protogynous flowering (Table 1). This treatment appeared to mimic the normal floral sequence seen under native conditions. Unchilled plants dug on 22 September 2011 and 6 October 2011 also displayed protogynous flowering, but unchilled plants dug on 20 October 2011 and 4 November 2011 exhibited protandrous flowering. The unchilled plants from the latter two dates reached anthesis prior to stigma appearance while the inflorescence culm was only 7.6 and 6.4 cm on average (Table 1). The reversal in dichogamy in the unchilled plants may be due to their longer exposure to short days and colder temperatures. Unchilled plants from the last two dates would have naturally received more chilling hours (under normal colder Minnesota days) than plants dug on earlier dates. Plants dug on 22 September 2011, 6 October 2011, 20 October 2011, and 4 November 2011 would have been exposed to 10, 20, 27, and 41 nights respectively when low temperatures reached 10 °C or less (Minnesota Climatology Working Group, 2012). It is possible that unchilled plants dug on the latter two dates had already begun going dormant for winter and required an extended chilling treatment to either overcome delayed (but not arrested) floral development or to cause the inflorescence culm to elongate. This result is consistent with a study of 9 British *Carex* species that required a chilling treatment after going dormant in November to stimulate inflorescence growth (Smith 1966).

Carex pensylvanica plants exposed to a post-induction chilling treatment produce protogynous flowering.

For genotype MN101B, *C. pensylvanica* is induced by short day photoperiods and then a subsequent chilling treatment was shown to stimulate rapid inflorescence culm elongation and to promote the protogynous sequence of flowering. Plants in all four chilling treatments exhibited protogynous flowering regardless of the temperature during floral induction or floral development. In contrast, unchilled plants flowered protandrously.

We cannot call the post-induction chilling treatment a dormancy-breaking treatment. Dormancy is defined as the "absence of visible growth in any plant structure containing a meristem" (Lang 1987). This definition of dormancy does not describe the floral meristem in *C. pensylvanica*. When given favorable growing conditions, the *C. pensylvanica* floral meristem is capable of producing an inflorescence with both pistillate and staminate flowers without a dormancy-breaking or chilling treatment. The problem is that a lack of a chilling treatment will result in a delay in floral development that manifests itself as slow inflorescence culm growth and a reversal in dichogamy. The chilling treatment is thus necessary to overcome this delay in floral development but is technically not a dormancy-breaking treatment.

Temperate herbaceous plants do not commonly undergo floral induction and initiation in late summer or fall and then rapidly proceed through floral development in early spring. This is a pattern more common of arctic or alpine plants that must rapidly flower and set seed after spring snowmelt to take advantage of a short growing season (Crawford 2008). *Carex pensylvanica* flowering is thus more similar to *C. bigelowii*, a

circumpolar arctic sedge that benefited from a post-induction chilling treatment to increase the percentage of flowering plants and to accelerate days to flowering (Heide 1992). This pattern of fall bud set and early spring flowering may seem unusual unless placed within the context of the genus. *Carex*, a genus of over 2000 species, does not display increased species richness at tropical latitudes like most genera (Escudero et al. 2012). Instead, *Carex* exhibits increased species diversity at higher latitudes and *Carex* clades appear to have diverged during periods of global cooling (Escudero et al. 2012). Fall bud initiation combined with early spring flowering would have been an advantageous reproductive trait as *Carex* spp. radiated from lower to higher latitudes.

Genotypic variation appears to exist within this species. Genotype MN102O was used to test whether a 10-week chilling treatment could overcome incomplete floral induction and still produce normal flowering. A chilling treatment did not enforce or promote normal floral induction in this genotype. Instead, a chilling treatment only overcomes delayed floral development in *C. pensylvanica*. It is likely MN102O plants were not sufficiently induced by 6 weeks of short day conditions under colder temperatures and therefore, the chilling treatment did not produce normal floral development. Abnormalities in staminate flowering in MN102O support this hypothesis. MN102O exhibited stigmas on the normally unisexual staminate spike (Fig. 6). In *C. flava,* the stigmas replaced the normally unisexual staminate spike when the plants were subjected to incomplete or marginal floral induction (Heide 2004). Thus, the flowering morphology of more than one *Carex* spp. is regulated by changes in environmental conditions during floral induction, initiation, and development.

Conclusion

Carex pensylvanica is a good model species for studying the effect of environmental factors on dichogamy. By manipulating post-induction temperature, one can reverse or restore the native sequence of flowering in this monoecious species. The key to producing protogynous flowering is to understand the life cycle. Like many Arctic plants, *C. pensylvanica* plants are determined and florally initiated by late fall. After plants are florally initiated, a subsequent chilling treatment or winter temperatures are necessary to produce rapid inflorescence elongation and normal flower development. Thus, dichogamy is a plastic trait that can be manipulated in *C. pensylvanica*.

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Table 1. The effect of date of removal from field and a chilling treatment on *Carex pensylvanica* flowering percent, average height at anthesis, average height at stigma appearance, average difference in height, average time to first flower and average difference in flowering dates.

Treatment	**Date**	**Flowering Percent**	**Average Height at Anthesis (cm)**	**Average Height at Stigmatic Appearance (cm)**	**Average Height Difference (cm)**	**Average Time to First Flower (days)**	**Average Difference in Flowering Dates**
Unchilled	Sept. 22, 2011	40	12.4 a ± 1.6	10.2 ab ± 0.36	2.17 b + 1.3	41.0 a ±3.3	0.867 b ± 1.2
Unchilled	Oct. 6, 2011	70	14.7 a ± 2.6	11.8 ab ± 2.2	2.93 ab + 0.48	37.0 a ±1.5	3.50 ab ± 0.29
Unchilled	Oct. 20, 2011	100	7.58 b ± 0.41	12.5 a ± 1.1	-4.92 c + 1.2	24.8 b ±1.6	-5.85 c ± 1.2

Unchilled	Nov. 4, 2011	90	6.38 b ± 0.29	10.5 ab ± 1.1	-4.11 c + 1.1	17.2 c ±0.60	-4.64 c ± 1.4
Chilled	Sept. 22, 2011	100	15.2 a ± 0.31	8.89 b ± 0.38	6.3 a + 0.27	13.9 c ±0.50	4.37 ab ± 0.25
Chilled	Oct. 6, 2011	100	14.2 a ± 0.85	9.65 ab ± 0.38	4.56 ab + 0.59	14.9 c ±0.38	5.32 a ± 0.73
Chilled	Oct. 20, 2011	90	13.5 a ± 0.78	9.88 ab ± 0.45	3.60 ab + 0.54	14.0 c ±0.83	2.66 ab ± 0.23
Chilled	Nov. 4, 2011	100	14.3 a ± 0.64	12.5 ab ± 0.40	1.77 b + 0.71	13.1 c ±.57	1.32 b ± 0.56

Note: Mean separation was not calculated for flowering percentage due to the lack of replication. Mean separation was calculated separately for each column. Values labeled with different lowercase letters were significantly different according to Tukey's honest significant difference test at $P \leq 0.05$.

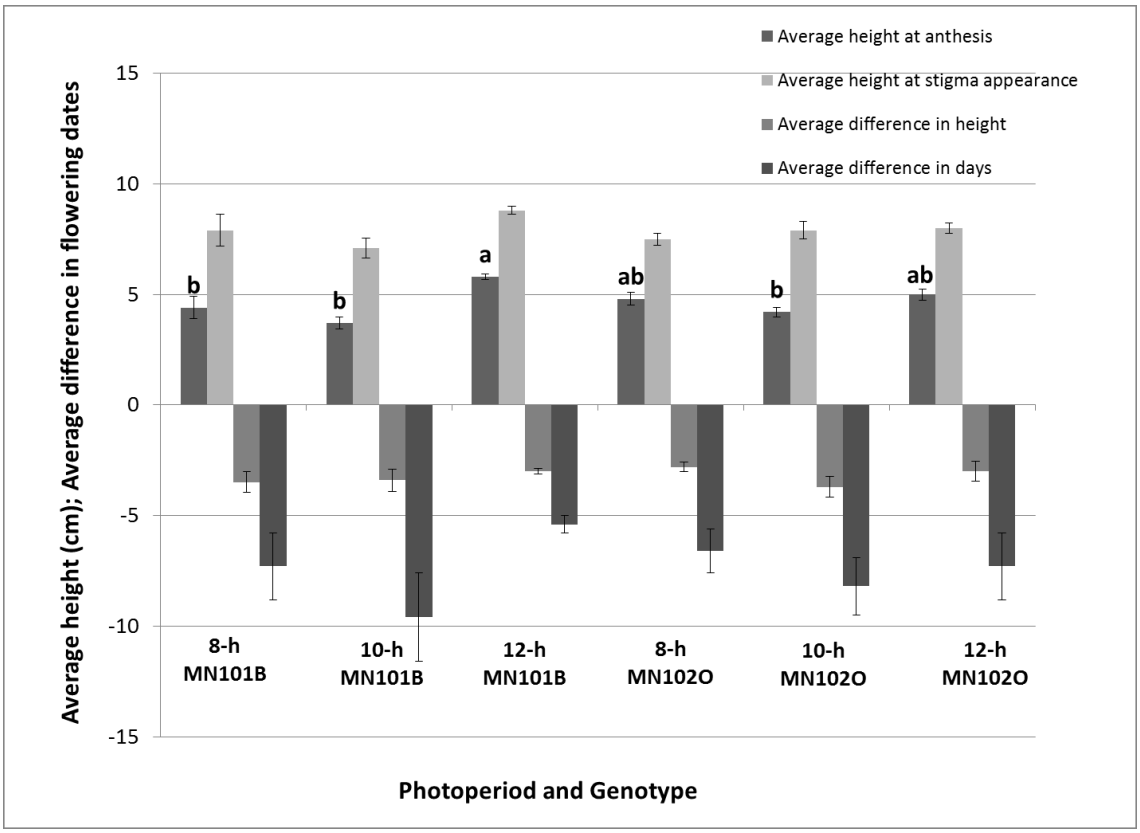

Fig 1. Effect of photoperiod on two *C. pensylvanica* genotypes. From left to right, the bars show average height at anthesis, average height at stigma appearance, and average difference in height (defined as the average difference in inflorescence culm height at stigma appearance and at anthesis) in cm. The fourth set of bars show average difference in days between time to stigma appearance and time to anthesis. Negative values for average difference in height and average difference in days indicate protandrous flowering. Only average height at anthesis was significantly different for the two genotypes. Bars labeled with different letters were significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. Standard error of the mean is indicated by error bars.

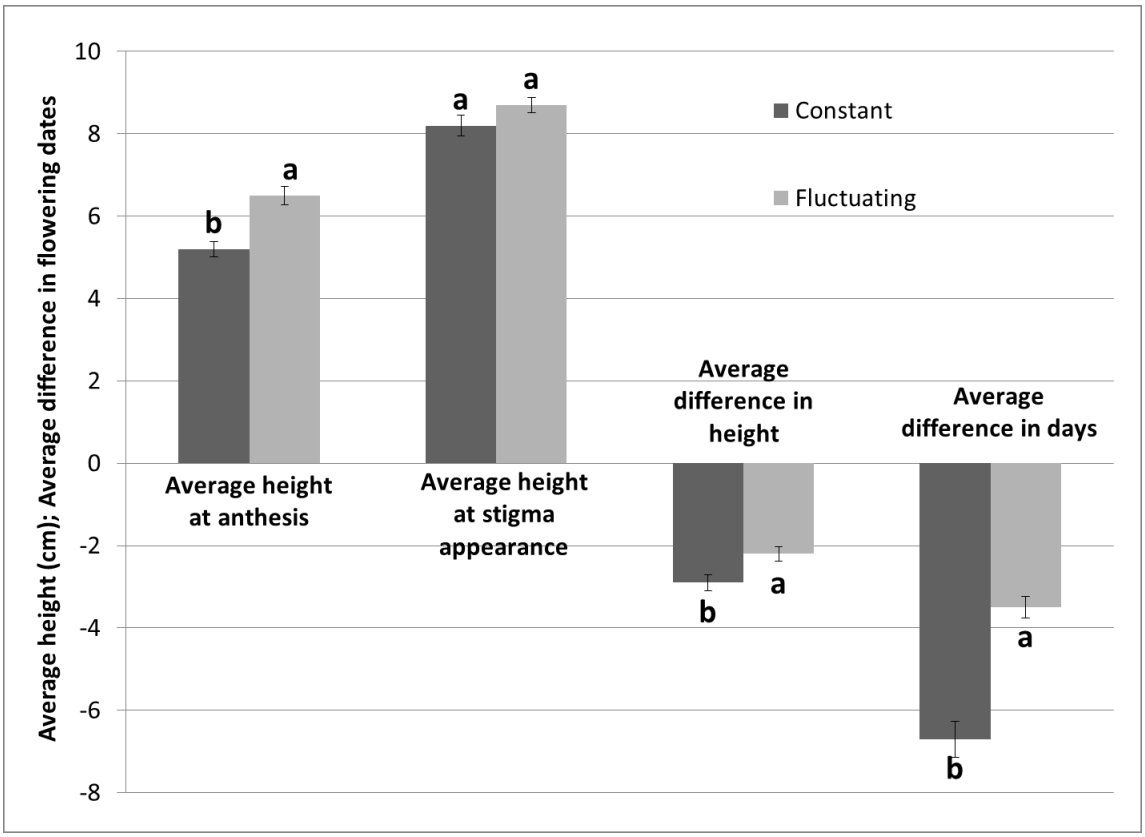

Fig 2. The effect of constant versus fluctuating temperature on *C. pensylvanica* average height at anthesis, average height at stigma appearance, and average difference in height. The fourth set of bars show average difference in days between time to stigma appearance and time to anthesis. Negative values for average difference in height and average difference in days show protandrous flowering. Bars labeled with different letters were significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. Standard error of the mean is indicated by error bars.

Fig. 3. Photograph of an immature *C. pensylvanica* inflorescence collected from field-grown plants on November 18, 2011. Black line equals 0.5 cm.

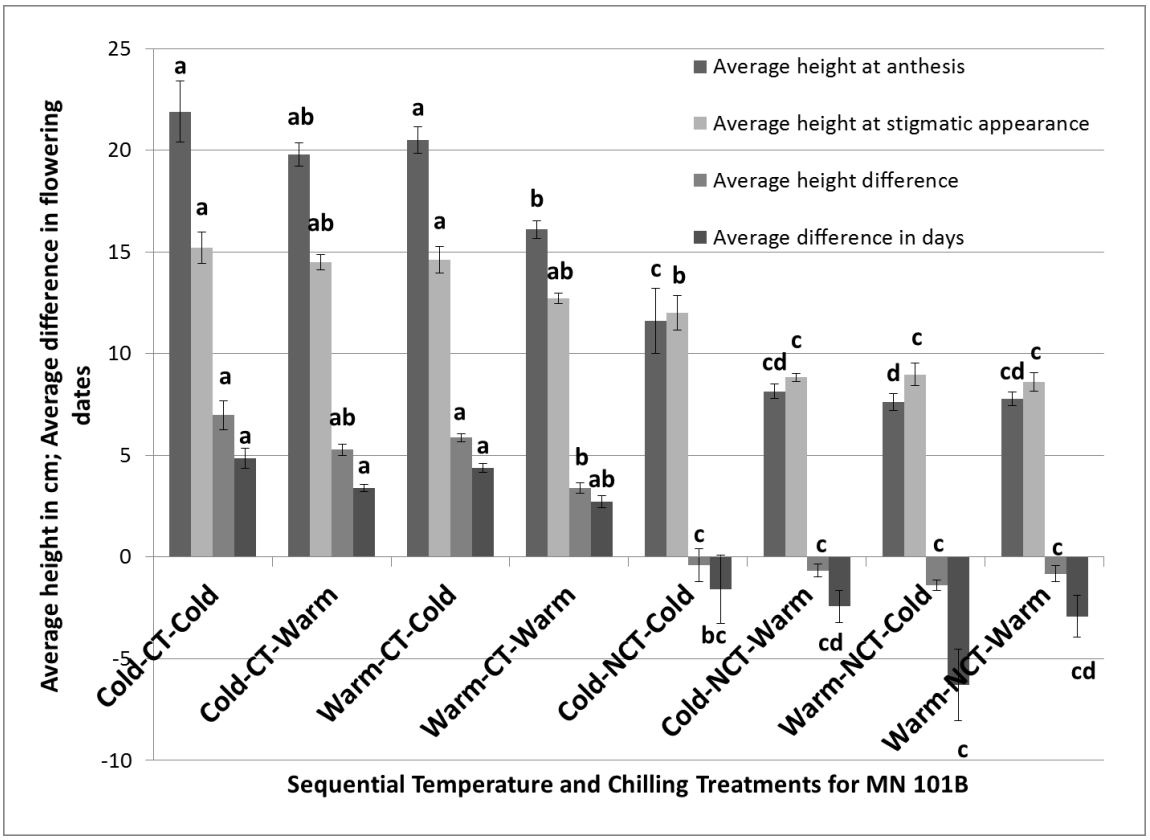

Fig. 4. The effect of sequential temperature and post-induction chilling treatments on flowering in *C. pensylvanica* genotype MN101B. CT stands for chilling treatment and NCT stands for no chilling treatment. The bars show from left to right average height at anthesis, average height at stigmatic appearance, and average height difference in cm. The fourth set of bars indicates the average difference in days between time to stigmatic appearance and time to anthesis. Positive values for average height difference and average difference in days show protogynous flowering and negative values show protandrous flowering. Bars labeled with different letters were significantly different

according to Tukey's honest significant difference test at $P \leq 0.05$. Standard error of the mean is indicated by error bars.

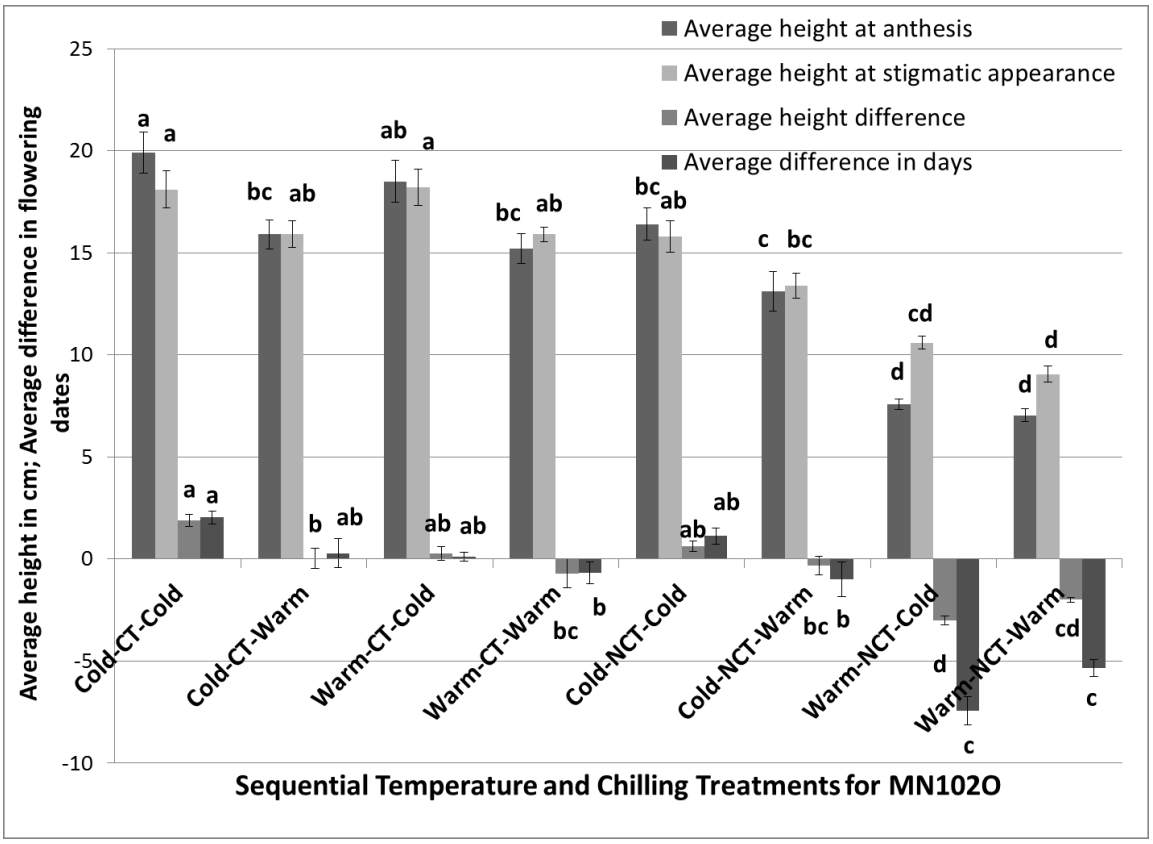

Fig. 5. The effect of sequential temperature and post-induction chilling treatments on flowering in *C. pensylvanica* genotype MN102O. CT stands for chilling treatment and NCT stands for no chilling treatment. The bars show from left to right average height at anthesis, average height at stigmatic appearance, and average height difference in cm. The fourth set of bars indicates the average difference in days between time to stigmatic appearance and time to anthesis. Positive values for average height difference and average difference in days show protogynous flowering and negative values show protandrous flowering. Bars labeled with different letters were significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. Standard error of the mean is indicated by error bars.

Fig. 6a and b. The top photo depicts protogynous (top) and protandrous (below) flowering in *Carex pensylvanica*. In protogynous flowering, the white stigmas on the lateral spikes appear first. In protandrous flowering, the yellow stamens on the terminal spike appear first. The bottom photo shows abnormal flowering in genotype MN102O. Note the white stigmas intermixed with the stamens on the terminal spike. The black line in both photos equals 0.5 cm.

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