ELSEVIER

Field Crops Research 75 (2002) 97–110

Field Crops Research

www.elsevier.com/locate/fcr

Physiological genetics of alfalfa improvement: past failures, future prospects

J.J. Volenec[a,*], S.M. Cunningham[a], D.M. Haagenson[a], W.K. Berg[a], B.C. Joern[a], D.W. Wiersma[b]

[a]*Department of Agronomy, Purdue University, West Lafayette, IN 47907-1150, USA*
[b]*Marshfield Agricultural Research Station, University of Wisconsin, 8396 Yellowstone Dr., Marshfield, WI 54449, USA*

Received 3 February 2002; accepted 7 February 2002

Abstract

The objective of this paper is to assess the effectiveness of alfalfa (*Medicago sativa* L.) improvement efforts over the last century, and with the advent of molecular biology, identify challenges for alfalfa improvement in the future. Yield trials conducted between 1986 and 1998 from around the US were used to compare yield and persistence of older alfalfa cultivars to those released in the 1990s. First and second harvest forage yield of recently released alfalfa cultivars were not improved over those of older cultivars. New cultivars had higher forage yield at fourth harvest, in early September, possibly due to a reduction in fall dormancy. Efforts to improve alfalfa persistence by breeding for improved disease resistance and greater winter hardiness also have not been effective at most locations. Use of molecular biology for alfalfa improvement depends upon identifying genes that control important agronomic traits that translate into greater yield, improved persistence, and enhanced forage quality. Few such genes have been identified in alfalfa, and their use might be complicated by the polyploid nature of this outcrossing species. The *Medicago truncatula* genome project is providing large amounts of sequence information, but little is known about the regulation of these genes and the function of their protein products *in planta*. Uncertainty exists regarding the effectiveness of transferring these genes to alfalfa to obtain a desired phenotype. Much remains to be done to identify key genes that determine agronomic performance of crop plants, including alfalfa, and to clarify mechanisms that regulate the expression of genes and the function(s) of their protein products under field conditions. Future efforts to improve agronomic performance of alfalfa will be enhanced by partnerships between public and private scientists because companies now dominate commercial release of new alfalfa cultivars.

Keywords: Alfalfa; β-Amylase; Chitinase; Disease resistance; Genetic improvement; Genomics; *Medicago sativa*; Persistence; Stress tolerance; Vegetative storage protein; Winter hardiness

1. Introduction

Alfalfa (*Medicago sativa* L.) is extensively used as a forage legume with over 32 million hectares grown worldwide and 15 million hectares in North America (Barnes et al., 1988; Michaud et al., 1988). Genetic improvement of alfalfa has been ongoing in the US for nearly a century following the initial field evaluations of ''Grimm'' alfalfa in Minnesota in 1901. During the first 80 years of the 20th century the rate of new cultivar release was slow, being largely dependent on public breeding programs with a few new cultivars

* Corresponding author. Tel.: +1-765-494-8071; fax: +1-765-496-2926.
E-mail address: jvolenec@purdue.edu (J.J. Volenec).

0378-4290/02/$ – see front matter
PII: S0378-4290(02)00020-5

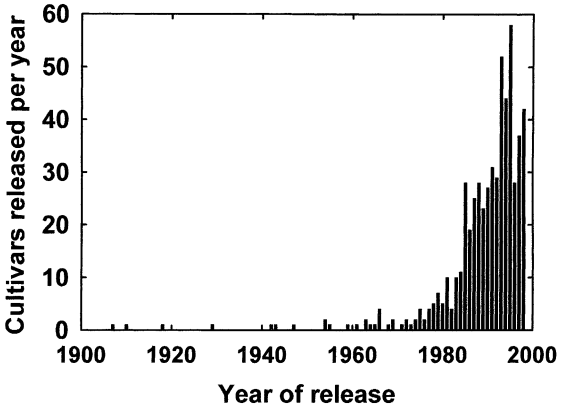

Fig. 1. Number of alfalfa cultivars released per year from 1900 to 1998.

released each decade (Fig. 1). In the late 1970s, a rapid increase in release of new cultivars began, especially from the private sector, with 27 or more new cultivar releases each year from 1990 to 1998. Does this large annual influx of new cultivars reflect rapid advancement in alfalfa performance due to plant breeding and/or application of new technologies in cultivar development, or is it an indication of other forces that influence cultivar release? The objective of this paper is to assess the effectiveness of alfalfa improvement efforts over the last century, and with the advent of molecular biology, identify challenges for alfalfa improvement in the future.

2. Genetic improvement of alfalfa forage yield

2.1. Trends in forage yield potential during the 20th century

To evaluate alfalfa yield and persistence trends we used a database that contains over 200,000 individual observations of forage yield, forage quality, stand, and winter survival data from 715 alfalfa variety trials conducted by universities in the US from 1986 to 1998. The comprehensive nature of the database enables us to reduce the influences of environment and genotype × environment interactions on alfalfa performance, and focus on trends in genetic improvement for alfalfa yield and persistence. We examined yield data for three individual forage harvests (harvests 1, 2, and 4). Differences in number of harvests per year (2–9) made it impossible to compare total forage production per year. In the Midwest US where most of this research was conducted, harvest 1 occurs in late May or early June. Remaining harvests occur every 30–35 days thereafter, with harvest 4 typically occurring in September. For this comparison, we calculated the average harvest 1 yield of each cultivar in every trial where it was grown regardless of the length of the trial. Cultivars were then sorted by year of release, and the average harvest 1 yield for all cultivars released in that year was calculated (Table 1). Likewise, this approach was used to calculate the maximum yield observed in any year or environment for all cultivars released in a given year to assess temporal changes in genetic yield potential of cultivars (best performance observed at any location). Our assumptions include that the trials were conducted using best management practices in order to evaluate cultivar yield potential. This would include selection of fields adapted to alfalfa production, high soil fertility, control of pests, and proper cutting management.

Not surprisingly, few cultivars released before 1940 were included in alfalfa cultivar trials conducted between 1986 and 1998. This resulted in fewer observations of harvest 1 yield for these pre-1940 cultivars (Table 1), so we have less confidence in those data. In 1954, the cultivar Vernal was released and has served as a benchmark cultivar in most trials since its release. The average harvest 1 yield of Vernal (1348 observations into its mean) and Lahontan (52 observations into its mean) was 4.30 Mg/ha; a value similar to harvest 1 yield of cultivars released in the 1990s (decade average = 4.33 Mg/ha) (Table 1). Regression of harvest 1 yields versus year of release resulted in a significant linear relationship ($R^2 = 0.20, P < 0.05$) with a slightly negative slope (Fig. 2). This indicates that harvest 1 forage yields of recent cultivars are not higher, and may be slightly lower than that of older cultivars. This observation agrees with results of Holland and Bingham (1994) who reported that forage yield of Vernal (released in 1954) was similar to that of the cultivars released between 1979 and 1985.

Trends of maximum harvest 1 yields were similar to those for average yield (Fig. 2, slope $= 0, R^2 = 0$). The maximum harvest 1 yields observed for Vernal and Lahontan released in 1954 (7.62 Mg/ha, Table 1) were similar to the maximum harvest 1 yields observed for cultivars released in the 1990s (decade

Table 1
Summary of harvest 1 forage yield (dry matter basis) of alfalfa cultivars averaged over all location-years, and maximum yield observed at any location in any year (1986–1999) as influenced by year of cultivar release[a]

Year released	Cultivars	Average yield, harvest 1 (Mg/ha)	Highest yield, harvest 1 (Mg/ha)
1907	Cossack (2)	5.74	5.78
1910	Ladak (10)	4.44	7.62
1918	Sevelra (7)	6.28	7.62
1929	Orestan (3)	6.59	7.17
1942	Ranger (130)	4.51	11.88
1943	Buffalo (91)	3.25	6.08
1947	DuPuits (52)	4.75	8.52
1954	Lahontan, Vernal	4.30	7.62
1955	Rambler	5.52	7.85
1959	Cody	3.05	4.89
1961	Beaver	2.76	3.90
1963	Saranac, Travois	4.01	7.35
1964	Ladak-65	4.44	9.51
1965	Washoe	5.87	7.40
1966	4	4.75	7.15
1968	Kanza	5.31	9.37
1969	Moapa, Victory	3.16	4.98
1971	Drylander	5.22	7.22
1972	Agate, Rambler	4.39	6.50
1973	Arc	3.36	8.45
1974	Deseret, Lew	4.33	5.00
1975	4	3.72	6.84
1976	Baker, CUF 101	3.09	5.81
1977	4	3.95	6.50
1978	5	4.37	6.93
1979	7	3.90	6.34
1980	5	3.99	6.46
1981	10	4.78	7.24
1982	4	3.45	5.43
1983	10	4.19	6.61
1984	11	4.17	7.06
1985	28	4.10	7.65
1986	19	3.70	6.61
1987	25	4.19	7.73
1988	28	4.37	8.47
1989	23	4.15	7.98
1990	27	4.33	8.34
1991	31	4.28	8.54
1992	29	4.24	8.27
1993	52	4.51	8.41
1994	44	4.46	8.27
1995	58	4.30	7.51
1996	28	4.30	6.97
1997	37	4.37	7.24
1998	42	4.10	6.79

[a] Cultivar names are listed when two or fewer were released in the year indicated. Numbers in parentheses after cultivar names (up to 1947) indicate the number of harvest 1 observations that went into the means to illustrate the fewer observations for harvest 1 yield for cultivars released from 1907 to 1929.

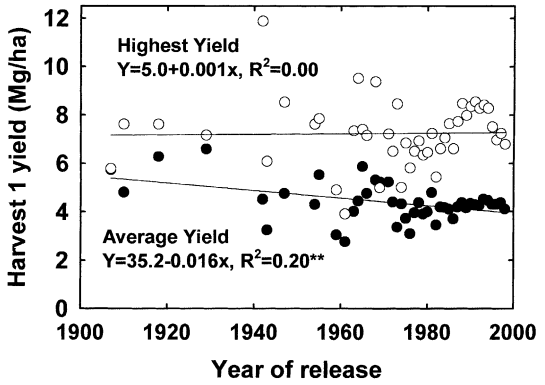

Fig. 2. Influence of year of cultivar release on harvest 1 forage yield of alfalfa. Average harvest 1 yields for cultivars released in a given year are averages over all location-years where those cultivars were tested between 1986 and 1998, whereas highest harvest 1 yield is that observed at any location-year between 1986 and 1998. Data were obtained from cultivar performance trials conducted in North America between 1986 and 1998 and are expressed on a dry matter basis. Data for a given year were averaged over all cultivars released within that year as listed in Table 1. Linear regression was used to determine trends in harvest 1 yields over time.

average of 7.82 Mg/ha; Table 1). This suggests that yield of alfalfa cultivars released in the 1990s in the "best" environments encountered between 1986 and 1998 was similar to earlier-released cultivars. Comparisons of average and maximum yields for harvest 2 produced similar conclusions to those reported for harvest 1 (data not shown).

Analysis of harvest 4 data produced similar results to those reported for harvest 1 forage yield. There was no trend over time for improvement in either average forage yield or maximum forage yield of alfalfa (Fig. 3). However, when data from cultivars released between 1978 and 1998 were selected for more detailed study, a trend for increased average yield and increased maximum forage yield was observed (Fig. 4). In this data subset cultivars released between 1978 and 1998 increased harvest 4 forage yield linearly at a rate of about 1.6% per year. Maximum forage yields increased in a quadratic fashion with rapid improvement occurring for cultivars released between 1980 and 1990. These positive trends in harvest 4 forage yields during the last 20 years were not observed in harvest 1 (Fig. 2) or harvest 2 (data not shown) data. The reason for improved harvest 4 forage yield is not known, but may be due to recent emphasis

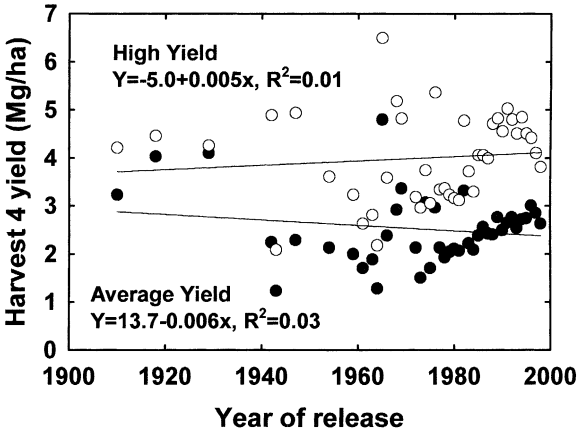

Fig. 3. Influence of year of cultivar release on harvest 4 forage yield of alfalfa. Average harvest 4 yields for cultivars released in a given year are averages over all location-years where those cultivars were tested between 1986 and 1998, whereas highest harvest 4 yield is that observed at any location-year between 1986 and 1998. Data were obtained from cultivar performance trials conducted in North America between 1986 and 1998 and are expressed on a dry matter basis. Data for a given year were averaged over all cultivars released within that year as listed in Table 1. Linear regression was used to determine trends in harvest 4 yields over time.

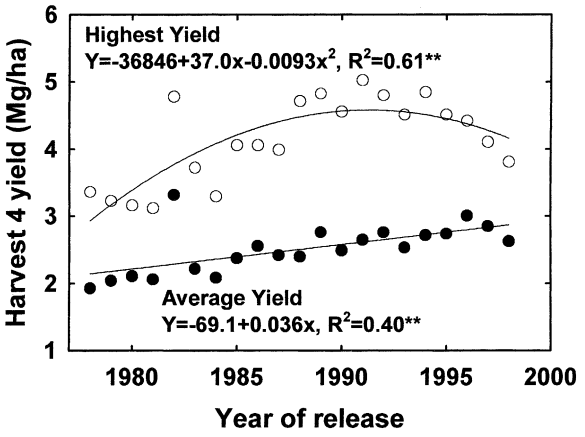

Fig. 4. Influence of year of cultivar release on harvest 4 forage yield of alfalfa. Average harvest 4 forage yield was averaged over all location-years where cultivars were tested between 1986 and 1998, whereas the highest harvest 4 yield is that obtained at any location-year between 1986 and 1998. Data were obtained from cultivar performance trials conducted in North America between 1986 and 1998 and are expressed on a dry matter basis. Data for a given year are averaged over all cultivars released in that year. For this analysis years of release were limited to 1978–1998 as listed in Table 1. Regression was used to determine trends in harvest 4 yields over time.

on release of less fall dormant cultivars that produce greater herbage growth in autumn. Greater fall dormancy has historically been used to identify more winter hardy plants because of the close positive correlation between these two traits (Barnes, 1991; Sheaffer et al., 1992). Plant breeders have attempted to reduce fall dormancy in newer cultivars, while maintaining high winter hardiness levels. The effect of selection for improved winter hardiness on alfalfa persistence will be discussed below.

Changes in total seasonal yield during various periods of the 20th century have been evaluated in two studies in Wisconsin. Holland and Bingham (1994) grew 12 alfalfa cultivars representing three selection eras (from 1898 to 1985) in rows for 3 years to determine the extent of genetic improvement for forage yield. Regression of yield versus year of release resulted in a positive linear relationship with a slope of 6.4 g per plot per year (0.18% per year). This indicates that alfalfa forage yield potential increased 15.7% over the 87-year period spanned by these cultivars. Ipson (1991) used 114 Wisconsin alfalfa variety trials conducted over a 30-year period from 1959 to 1989 to evaluate genetic improvement of forage yield. Seasonal yield increased an average of 0.5% per year and resulted in a 15% increase in forage yield potential being realized during the 30-year period of alfalfa improvement covered in this study. During this same period the state wide average for alfalfa yield per hectare in Wisconsin had increased 80% from 5.6 to 10.1 Mg/ha, indicating that improved management accounted for a 65% increase in alfalfa yield, which is equivalent to 77% of the total increase in yield. Neither study examined within-season changes in forage yield, so it is not known if the yield improvements they observed occurred in harvest 4 as we observed in this analysis (Fig. 4). In summary, although small increases in seasonal yield of modern alfalfa cultivars has occurred at some locations, part of this increase may be due to improved late-season growth of the less fall-dormant cultivars released during the last 20 years.

3. Breeding for improved alfalfa persistence

When considering genetic improvement of perennial crops like alfalfa, forage yield is one of the several

factors that should be considered. Another trait valued by both producers and alfalfa breeders is plant persistence because it improves stand longevity of this perennial species (Beuselinck et al., 1994). Many factors influence plant persistence and how alfalfa reacts to biotic and abiotic stresses. Two factors generally accepted as influencing alfalfa persistence include disease resistance and, in northern latitudes, winter hardiness. When information on winter survival is not available, fall dormancy has often been used to predict cultivar differences in winter hardiness (Barnes, 1991; Sheaffer et al., 1992).

3.1. Improving alfalfa persistence by selecting for greater disease resistance

At least six different pathogenic organisms can attack alfalfa causing the following diseases: anthracnose caused by *Colletotrichum trifolii* Bain and Essary; bacterial wilt caused by *Clavibacter michiganese* subsp. *insidiosum* (McCull) Davis et al.; fusarium wilt caused by *Fusarium oxysporum* Schlecht f. sp. *medicaginis* (Weimer) Snyd. and Hans.; verticillium wilt caused by *Verticillium albo-atrum* Reinke and Berth; phytophthora root rot caused by *Phytophthora megasperma* Drechs f. sp. *medicaginis*; and aphanomycetes crown and root rot caused by *Aphanomycetes euteiches* Drechs. Alfalfa producers, seed marketers, and university extension educators generally believe that alfalfa persistence is positively correlated with the level of resistance to these diseases (Holin, 2001). This hypothesis was examined using the cultivar data in the alfalfa cultivar performance database by comparing percent stand in the 4th or 5th year of forage production with disease resistance rating of each cultivar.

To facilitate regression analysis, numerical scores were substituted for the traditional letter scale used to rate disease resistance as follows: susceptible or no rating, 1; low resistance, 2; moderate resistance, 3; resistant, 4; highly resistant, 5. For each cultivar these numerical scores were summed over the six major diseases described above. For a cultivar that is reported to be highly resistant to all six diseases described above the scores would sum to 30, and these plants would be expected to have better persistence than cultivars with lower cumulative disease resistance scores. The results of this conversion from letter to numerical scores for one trial conducted in Grand Rapids, MN, are shown in Table 2. Scores ranged from a low of 11 for Vernal to a high of 29 for several of the newly released cultivars that possess high resistance to nearly all of the diseases described above. Linear regression of percent stand at this location against the sum of the disease resistance values was not significant (Fig. 5) indicating that things other than disease resistance controlled the fivefold range in percent stand at this location.

The regression results from the 37 cultivar trials reporting percent stand are summarized using a bubble graph (Fig. 6). This graph design permits three variables (slope, significance of the regression, and R^2) from the regression equations to be presented simultaneously. Nine of 37 trials gave the expected results of a significant ($P < 0.10$) positive slope with R^2 of approximately 0.20 or greater indicating that in these trials, cultivars with high disease resistance ratings had better stands than did cultivars with low disease resistance ratings. Four of 37 trials gave the unexpected results of a significant ($P < 0.12$) negative slope with R^2 of 0.12–0.18 indicating that cultivars with high disease resistance ratings had poorer stands than did cultivars with low disease resistance ratings. Most trials (24 of 37, 65%) showed no significant effect of disease resistance rating on percent stand. Ipson (1991) reported that disease limited alfalfa performance in only 10% of the 114 Wisconsin cultivar trials he evaluated. Similar to our findings, Ipson reported that greater disease resistance ratings were positively associated with increased persistence in a few trials, but also were associated with reduced persistence in others. He concluded that the unnecessary inclusion of high levels of disease resistance that are not limiting yield can actually reduce alfalfa forage yield potential.

While the value of disease resistance has been documented many times in environments where the disease organism is known to exist or is artificially applied to alfalfa plants (Elgin et al., 1988 and references cited therein), our data shows little improvement in percent stand in response to enhanced levels of disease resistance. This suggests that a critical level of disease incidences are not as widespread as generally believed, and that other factors contribute to genetic differences in alfalfa stand decline. In addition, the aboveground inspection that is often used to determine

Table 2
Numerical ratings of disease resistance scores (susceptible or no rating, 1; low resistance, 2; moderate resistance, 3; resistant, 4; highly resistant, 5), and winter survival (WS) ratings for alfalfa cultivars grown at Grand Rapids, MN[a]

Cultivar	Year of release	BW	FW	VW	AN	PRR	APH	Sum	WS[b]	Percent stand
Depend + EV	1995	5	5	5	5	5	4	29		31
DK-122	1988	5	4	4	5	5	1	24		23
DK-133	1991	5	5	4	5	5	4	28	4.0	25
Evolution	1992	5	5	4	5	5	4	28		24
GH-767	1994	5	5	4	5	5	4	28	3.0	28
GH-794	1993	5	5	4	5	5	4	28		10
GH-797	1994	5	5	5	5	5	4	29		45
Pio:5246	1991	5	5	4	5	5	3	27		40
Pio:5262	1987	5	3	2	1	4	1	16	2.3	33
Pio:5312	1993	5	5	5	5	5	4	29	2.7	31
Pio:5454	1991	4	5	3	5	5	2	24	2.2	16
Profit	1985	5	5	4	3	4	1	22		21
Proof	1993	5	5	4	5	5	4	28		16
Rushmore	1993	5	5	4	5	5	5	29	3.2	15
Sterling	1993	5	5	4	5	5	4	28	2.0	23
Ultraleaf-87	1992	5	5	4	5	5	4	28	3.7	13
Vernal	1954	4	3	1	1	1	1	11	1.9	34
Wintergreen	1994	5	5	5	5	5	4	29	2.5	45
Winterstar	1995	5	5	5	5	5	4	29	2.3	38

[a] Diseases are abbreviated as: BW, bacterial wilt; FW, fusarium wilt; VW, verticillium wilt; AN, anthracnose; PRR, phytophthora root rot; APH, aphanomycetes root rot. For each cultivar, ratings are summed over the six diseases to give a total disease resistance score (sum). Visual estimates of percent stand in year 4 of the trial are provided.

[b] Winter survival rating where 1: no injury, 6: dead plant.

percent stand may not be sufficiently accurate to detect differences in stand caused by greater disease resistance if they do in fact exist. Coutts et al. (2001) recently reported that aboveground inspection

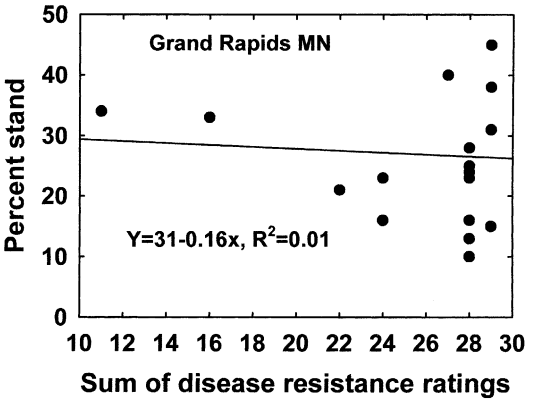

Fig. 5. Influence of disease resistance ratings on percent stand of alfalfa in the 4th production year at a cultivar performance trial established in 1994 at Grand Rapids, MN. Linear regression was used to evaluate trends in percent stand as influenced by disease resistance rating.

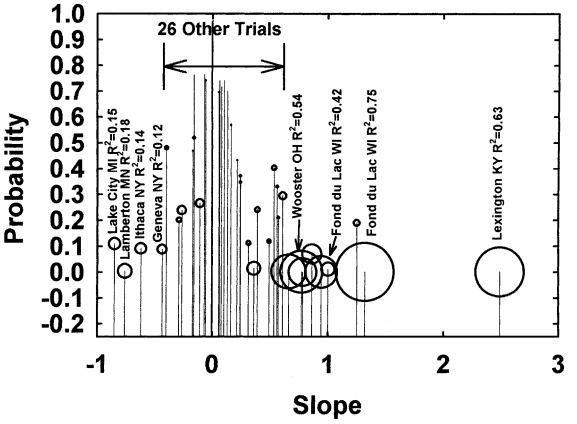

Fig. 6. Bubble plot summarizing linear regression information from 37 locations where percent stand and disease resistance ratings were reported in cultivar performance trials between 1986 and 1998. The *x*-axis presents the slope of the linear regression, whereas the *y*-axis indicates the probability that the slope is significantly different from 0. Diameters of the circles in the body of the graph indicate relative differences in the coefficient of determination (R^2). These R^2-values are provided for several locations. Drop lines to the horizontal axis are provided for all locations so data with low R^2-values are visible.

significantly underestimates alfalfa plants/m^2 because clusters of 2, 3, and 4 plants can be mistaken for a single plant, especially at high plant populations.

3.2. Selection for improved winter hardiness

Winter hardiness has been a second focus of scientists involved in alfalfa improvement. A standard test for winter survival has been developed (http://www.naaic.org/stdtests/wintersurvival.htm) and is used to categorize existing cultivars and develop new ones with improved winter hardiness. This test includes an early autumn clipping to enhance winter injury and more easily distinguish the most winter hardy plants within a breeding population. To determine the effectiveness of selecting for greater winter survival we compared percent stand in year 4 versus winter survival scores of each cultivar tested. Unlike disease resistance, not all cultivars had been evaluated for this trait (e.g., Table 2). The linear regression of percent stand in year 4 versus winter survival rating for one trial conducted at Arlington, WI, is shown in Fig. 7. Percent stand would be expected to decline with increasing winter survival ratings (slope should be negative), however, in this trial there was no relationship between these two variables. As with disease resistance, a bubble graph was used to summarize regression data for the 39 trials used to evaluate this relationship (Fig. 8). Regressions from four trials

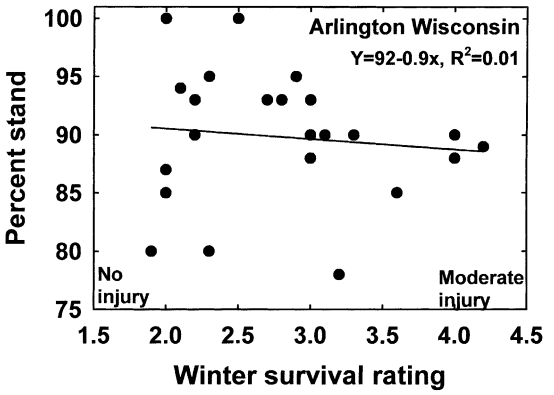

Fig. 7. Influence of winter survival ratings on percent stand of alfalfa in the 4th production year at a cultivar performance trial established in 1994 at Arlington, WI. Linear regression was used to evaluate trends in percent stand as influenced by winter survival rating.

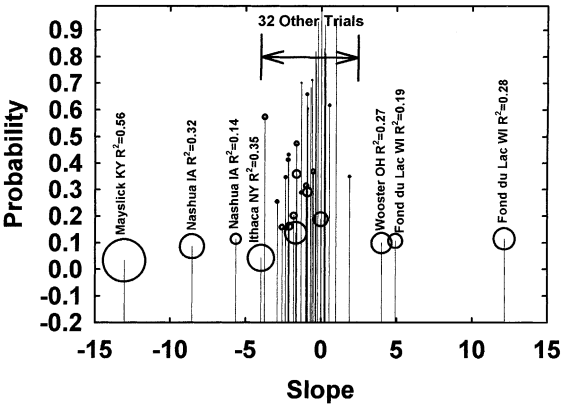

Fig. 8. Bubble plot summarizing linear regression information from 39 locations where percent stand and winter survival ratings were reported in cultivar performance trials between 1986 and 1998. The x-axis presents the slope of the linear regression, whereas the y-axis indicates the probability that the slope is significantly different from 0. Diameters of the circles in the body of the graph indicate relative differences in the coefficient of determination (R^2). These R^2-values are provided for several locations. Drop lines to the horizontal axis are provided for all locations so data with low R^2-values are visible.

had significant ($P \leq 0.12$) negative slopes and R^2 ranging from 0.14 to 0.56 (large bubbles left of slope = 0). Surprisingly, the most negative slope, and therefore the trial where low numerical scores for winter survival (e.g., high winter hardiness) were closely associated with percent stand was located in Mayslick, Kentucky, a location where severe winters are not likely to occur. Three trials exhibited the unexpected response of less percent stand decline being associated with lower winter survival scores. Two of these trials occurred at Fond du Lac, Wisconsin, where improved winter hardiness would be expected to improve percent stand. Like disease resistance ratings, however, differences in percent stand were not associated with winter survival ratings in most trials (32 of 39, 82%). Nearly all (36 of 39) trials used in this analysis were located in states where winter can be a severe abiotic stress (Michigan, Minnesota, Iowa, New York, Wisconsin, Pennsylvania, North Dakota, Ohio), with only three trials from regions with relatively mild winters (Oklahoma and Kentucky). Results from our analysis of these cultivar trials strongly suggests that selection for enhanced winter survival using current methods has not been effective for increasing alfalfa persistence. Effective

selection for winter survival is difficult because each winter is unique varying in many things including the severity of the low temperatures encountered and their duration, extent of snow cover, ice sheeting, intermittent warm periods that de-harden plants, and other uncontrollable environmental factors.

From our analysis of yield and persistence using this alfalfa cultivar performance data set, it is clear that progress toward improving alfalfa performance has been limited. Furthermore, traits currently being used as selection criteria are not very effective in developing alfalfa cultivars with the desired traits when plants are later evaluated under the conditions of these cultivar trials. With the advent of molecular tools and functional genomics, opportunities exist to genetically modify alfalfa by inserting specific genes that may improve agronomic performance. However, we must know what these genes are in order to realize this opportunity.

4. Future prospects: functional genomics and alfalfa improvement

4.1. GenBank and alfalfa improvement—what alfalfa genes are available?

While we possess the technology for inserting one or more genes into alfalfa, there is a great uncertainty regarding what specific genes to clone and insert into alfalfa (and other crops) to improve agronomic performance. With a relatively poor understanding of what gene(s) control growth, yield, stress tolerance, etc. the traditional approach has been to use genes currently available to conduct model studies rather than take on the difficult task of deciphering the genetic and physiological mechanisms controlling complex traits of agronomic value. Genes available for use include those already in alfalfa whose promoter could be altered to permit user-directed regulation. Genes from other organisms that, when correctly expressed, create new genetic variation also have potential for altering alfalfa phenotypes.

To better understand currently available alfalfa genes whose expression could be altered in useful ways, we searched GenBank using the keyword ''alfalfa''. As of June 2000, GenBank contained 476 accessions describing alfalfa ''genes''. After careful inspection this total was reduced, because it was necessary to remove 152 duplicate submissions (including instances where the entry was listed using two or more different names) and 52 cDNA fragments that would not be useful for cloning. This reduced the total number of alfalfa genes actually available for use to 272, or 57% of the number initially obtained from our GenBank search. Of these, 54 accessions were genomic sequences, and 28 of these were transposable elements. Because of tissue-specific gene expression and the subsequent variation in stability of transcripts and resultant proteins, the source tissue used for isolation of full-length cDNAs can be an important consideration.

Most of the alfalfa cDNAs in GenBank were obtained from two systems; cultured cells (33%) and root nodules (25%) (Table 3). Surprisingly, low percentages of the alfalfa cDNAs were obtained from leaves (14%), roots (7%), and stems (3%). While sequences of cDNAs isolated from cultured cells can be identical to those found in roots, stems, and leaves, they also can be very different. For example, seven mitogen-activated protein kinase (MAP-kinase) accessions are in GenBank for alfalfa. A comparison of sequence homology indicates that two of these (S4812 and X6646) are identical to each other and to MAP-kinase cDNAs isolated from nodules and roots (Fig. 9). However, two other MAP-kinase cDNAs from cultured cells that are identical to each other share about 70% nucleotide homology with those isolated from roots and nodules. Finally, the MAP-kinase genomic clone reported for alfalfa

Table 3
Tissue sources used to isolate the 218 non-redundant, full-length cDNAs reported in GenBank for alfalfa as of June 2000

Tissue	Number of cDNAs
Cultured cells	73
Nodules	54
Leaf	31
Root	15
Seedlings	12
Stem	7
Meristem	7
Pollen	5
Seed	1
Crown	1
Unknown	12

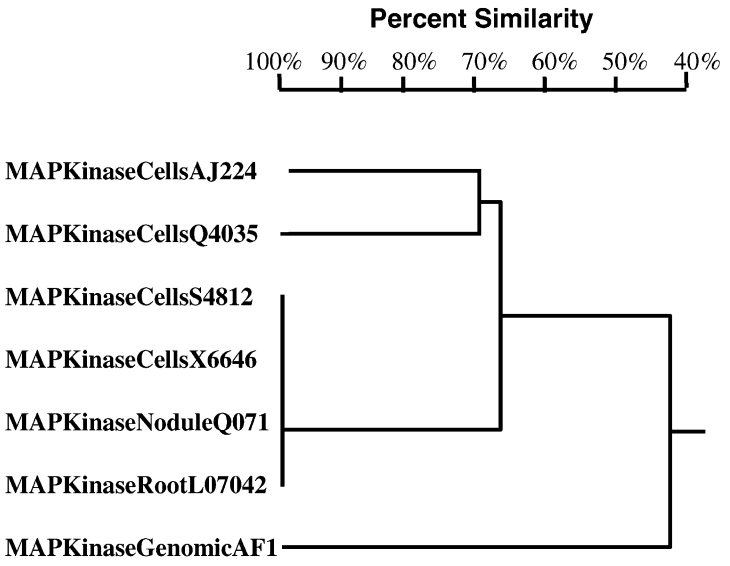

Fig. 9. Homology tree for MAP-kinase nucleotide sequences for alfalfa entries reported in GenBank as of June 2000. Percent similarity values are indicated.

possesses the lowest homology, with less than 50% homology with the MAP-kinase cDNAs from nodules and roots. Experiments demonstrating equivalent MAP-kinase function irrespective of this sequence diversity have not been reported. Therefore, it is not clear how this sequence diversity would impact efforts to clone distantly related MAP-kinases into specific tissues within a species (i.e., J224 into roots).

In addition to having relatively few alfalfa cDNAs reported in GenBank, accessions that are available were submitted from a small number of laboratories specializing in certain aspects of alfalfa biology. For example, over one-half of the alfalfa cDNAs reported in GenBank are sequences coding for proteins that function in secondary metabolism (34 accessions), nitrogen metabolism (27 accessions), stress tolerance (25 accessions), and cell division (25 accessions). In addition, within each of these four groups a high amount of functional duplication exists. For example, 10 of 34 sequences in the secondary metabolism group code for chalcone synthases, while seven of 25 accessions in the cell division group code for MAP-kinases, with only three different sequences being present (Fig. 9). As a result, we effectively have very few genes at our disposal from alfalfa per se for use in molecular crop improvement. This makes the *Medicago truncatula* genomics project (and other sequencing projects) an attractive alternative as a source of genetic variation for alfalfa improvement.

4.2. The M. truncatula genomics initiative—genes for the future?

The long-term goal of the *M. truncatula* genomics initiative is to compare genes of agronomic and scientific interest in *M. truncatula* to those found in related crop legumes including alfalfa (NSF Award Abstract #9872664; https://www.fastllane.nsf.gov/servlet/showaward?award=9872664). The hope is that this database of expressed genes will enable detailed analysis of the role of specific genes in plant growth and development. This knowledge will enable more efficient cloning and characterization of valuable genes and traits, such as disease resistance and crop productivity, and it will ultimately facilitate the development of improved crop cultivars.

As of 21 December 2000, the *M. truncatula* genome database contained 88,906 sequences, mainly as fragments of cDNAs known as expressed sequence tags (ESTs) (http://www.tigr.org/tdb/mtgi/). Of these 30,145 were judged as being unique. This is a tremendous catalog of genetic variation, but assigning function to these genes so that they can fulfill their

promise of utility in alfalfa improvement programs will require substantial effort and resources.

To illustrate the complications that might be encountered as protein function and molecular genetics are combined to create ''functional genomics'', we examined the role of chitinase in *M. truncatula* and alfalfa. Chitinases are hydrolytic enzymes that cleave β-1,4-glycosidic bonds between *N*-acetyl glucosamine monomers in chitin, the primary structural polysaccharide in cell walls of fungi, including many plant pathogens. The role of chitinases in plant defense responses to fungal pathogens has been frequently documented (Collinge et al., 1993). Six chitinase classes (I–VI) exist based on protein and nucleotide sequences (Levorson and Chlan, 1997). Recently, Salzer et al. (2000) reported that expression of classes I, II, and IV chitinase genes was enhanced during pathogen invasion. In contrast, class III chitinases were not enhanced during pathogen invasion, but were strongly induced in *M. truncatula* roots as they formed symbiotic associations with arbuscular mycorrhiza that assist with nutrient uptake. These authors concluded that expression of these class III chitinase genes could be considered a hallmark for the establishment of arbuscular mycorrhiza in *M. truncatula*. Thus, chitinases in *M. truncatula* have multiple physiological roles; one to ward off pathogen invasion, and the other to facilitate a symbiotic relationship with a soilborne fungus.

In alfalfa, chitinase has yet another function; as a vegetative storage protein (VSP). Four VSPs accumulate to high concentrations (>30% of the protein pool in alfalfa roots) specifically in taproots, and are used as a source of N for shoots when growth is initiated in spring (Fig. 10) and when it resumes after defoliation in summer (Avice et al., 1996; Barber et al., 1996; Cunningham and Volenec, 1996, 1998). N-terminal sequence analysis, and cloning and sequencing of cDNAs for the high molecular weight VSP (HMW VSP) confirm high sequence homology between this VSP and chitinases from several sources including *M. truncatula* (Fig. 11). The amino acid sequence on the HMW VSP upstream from the box containing the N-terminal sequence has been identified as a signal

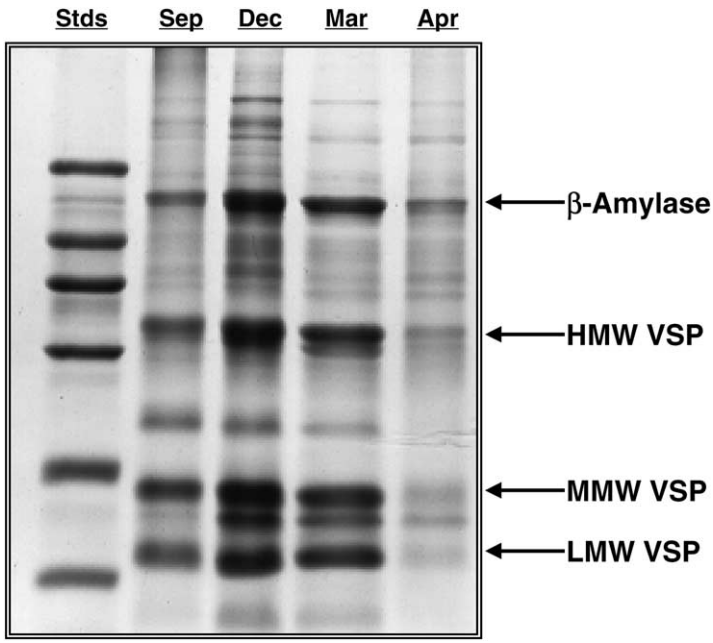

Fig. 10. Denaturing gel electrophoresis of proteins from alfalfa taproots sampled in September, December, March, and April showing changes in the relative abundance of β-amylase, and three other vegetative storage proteins (VSPs). These four polypeptides accumulated to high concentrations in taproots in autumn, and disappeared from taproots when shoot growth resumed in April. The gel was stained with Coomassie Brilliant Blue. Molecular weight standards are shown in lane 1 (Stds).

Fig. 11. Predicted amino acid sequence of a class III chitinase isolated from *M. truncatula* (MGI:S:420) and the predicted amino acid sequence of a clone for high molecular weight VSP from alfalfa taproots (Ms:HMW:VSP). The boxed sequence identifies the N-terminal sequence used to identify the VSP cDNA. Sequence preceding the boxed N-terminal sequence has been identified as a signal peptide.

sequence using Signal-P (Nielsen et al., 1997). Immunoblot analysis of proteins extracted from *M. truncatula* roots using antisera raised to alfalfa taproot VSPs detected small quantities of taproot VSP in one of the two cultivars studied (Cunningham and Volenec, 1996). This indicates that proteins similar to these alfalfa taproot VSPs can be found in *M. truncatula* roots, but their function as a VSP in *M. truncatula* has not been demonstrated.

Although possessing high amino acid homology between alfalfa and *M. truncatula*, the role of chitinases in this genus appears to be diverse, including pathogen defense, facilitating symbiosis, and N storage. Multiplicity of protein use in alfalfa is not unique to chitinases. We have shown that β-amylase also serves as a storage protein in alfalfa roots, along with its more traditional role in starch degradation (Fig. 10) (Boyce and Volenec, 1992; Gana et al., 1998). This indicates that, while sequence information may provide insight into what catalytic activity a protein may possess, it does not necessarily determine the role of the protein *in planta*. In other words, structure does not necessarily define function. Though this is a challenging concept to accept because it is contrary to existing paradigms, there is a growing awareness that it is presumptuous to think in terms of singular function and to make functional assignments merely on the basis of some degree of similarity between sequences. Attwood (2000) stressed the importance of combining structural, functional, and evolutionary studies to elucidate protein function, and that protein function is context dependent. Clearly, this is true for alfalfa where enzymes involved in fungal resistance and starch degradation in other biological systems serve as VSPs in alfalfa taproots.

4.3. Functional biology of alfalfa—the missing link

A major hindrance to the use of molecular tools for alfalfa improvement is that little is known about the physiological and biochemical mechanisms that control growth, yield, stress tolerance, forage quality, and other agronomic traits of this species. Even though we have an 80-year history of studying winter survival, we find our understanding is far from complete. For example, defoliating alfalfa in early autumn (15 September–15 October) in the Upper Midwest USA usually increases winter injury (Smith, 1972; Sheaffer et al., 1988 and references cited therein), especially if regrowing shoots are killed by frost before root carbohydrate reserves re-accumulate. However, cutting during this time may have no impact on winter hardiness, especially if autumn-like conditions conducive for regrowth continue for 6–8 weeks after cutting. This period of extensive regrowth allows plants to re-accumulate sugars and starches in roots, and the plant properly acclimates for winter. Sugar accumulation in alfalfa roots during autumn also has been consistently associated with genetic differences in winter survival (Castonguay et al., 1995; Cunningham and Volenec, 1998; Cunningham et al., 1998, 2001).

To determine if taproot sugar depletion in roots after autumn cutting reduces winter survival we defoliated field plots in early October that contained six alfalfa

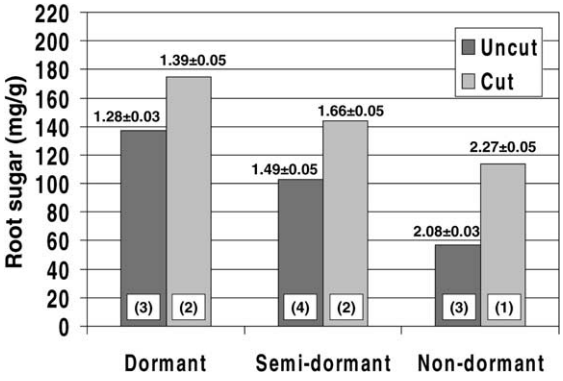

Fig. 12. Influence of defoliation in mid-October (cut) on winter injury rating (values above bars ± standard error) and root sugar concentrations (standard errors in boxes inside bars) in December of alfalfa cultivars differing in fall dormancy. Winter injury ratings are weighted averages for 30–40 plants where 1: no injury, 2: injured, and 3: dead. Data were averaged over 2 years.

cultivars ranging in fall dormancy (and therefore perceived winter hardiness) from three (dormant, winter hardy) to nine (non-dormant, not winter hardy). Using roots sampled in early December from both defoliated and intact plants, we compared sugar accumulation and expression of genes associated with winter hardiness (Haagenson et al., 2000). As expected, autumn defoliation increased winter injury in both years of the study, and the semi-dormant and non-dormant cultivars were damaged more than were the fall dormant cultivars (Fig. 12). To our surprise, however, defoliation in early October consistently increased sugar concentrations in taproots of all six cultivars in both years of the study (Fig. 12).

We also determined the expression of genes that we (Cunningham et al., 2001; Gana et al., submitted for publication) and others (Monroy et al., 1993) have shown to be consistently associated with genetic differences in winter hardiness. Our hypothesis was that mid-October defoliation interrupts cold acclimation, including expression of these cold acclimation responsive (CAR, CAS15a,b) genes. As expected, dormant and semi-dormant plants left intact throughout the autumn (Fig. 13, U-treatments) consistently had higher CAR gene expression in December than in October, and both of these groups had higher CAR gene expression than did 5939, a non-dormant cultivar that is not winter hardy (Fig. 12). Contrary to our hypothesis, defoliation in mid-October did not reduce CAR transcript levels when roots were sampled in December, even though defoliated plants had greater winter injury (Fig. 12). These results are clearly inconsistent with the generally accepted roles of sugar accumulation and CAR gene expression have in cold acclimation and winter survival. This indicates that careful re-evaluation of winter hardiness mechanisms is necessary before we begin to genetically engineer alfalfa for higher accumulation of root sugars to improve winter hardiness.

Even when a gene with a relatively simple ''function'' (based on the sequence analysis) such as nutrient uptake is identified, its function *in planta* may not be completely understood. For example, we have examined how expression of high affinity phosphate (P) transporters in alfalfa roots is influenced by P nutrition under field conditions (for a review of these P-transporters see Raghothama, 1999). Our experimental site

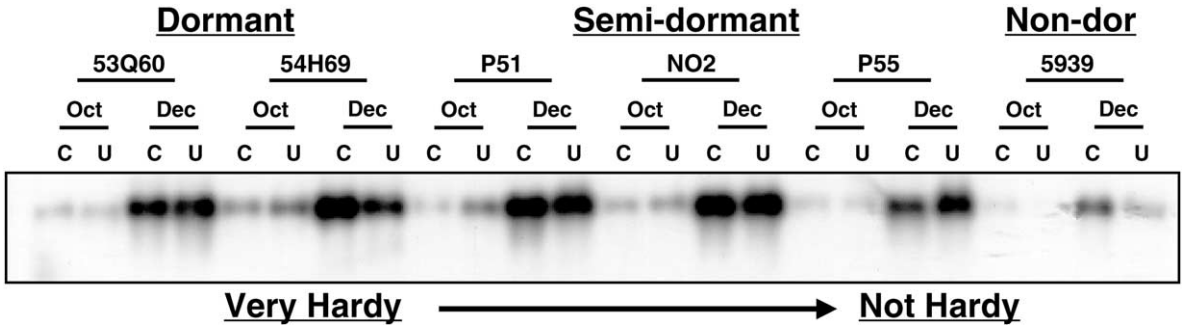

Fig. 13. Influence of defoliation in mid-October (cut: C, uncut: U) on expression (northern analysis) of cold acclimation-responsive genes (RootCAR1) in December in roots of alfalfa cultivars differing in fall dormancy. Two dormant cultivars (Pioneer 53Q60, Pioneer 54H69), three semi-dormant germplasms (P51, NO2, P55), and a non-dormant cultivar (Pioneer 5939) used in this study are identified.

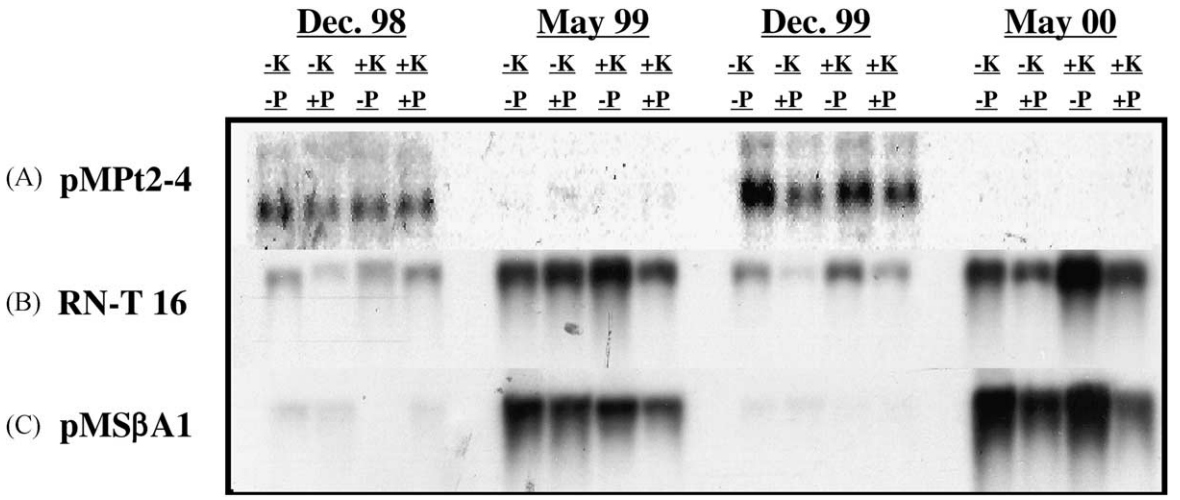

Fig. 14. Influence of potassium (K) and phosphorus (P) fertility on gene expression (northern analysis) in alfalfa roots. Field-grown plants were fertilized with K (+K = 400 kg K/ha per year) and/or P (+P = 75 kg P/ha per year), or were left unfertilized (−K, −P). Roots were sampled in the months shown and the isolated RNA probed with cDNAs corresponding to a high affinity P-transporter (pMPt2-4), the high molecular weight VSP (RN-T-16), and β-amylase (pMSβA1).

has very low soil P (≤5 ppm Bray P_1); a condition ideal for detecting high P-transporter expression. As expected, expression of these P-transporter genes was higher in alfalfa roots provided no P fertilizer, especially under low-K conditions (Fig. 14, pMPt2-4). To our surprise, however, this increased expression was not observed in May, but was in December when soil temperature was near 0 °C. Very little transcript for these P-transporters could be detected in May samples irrespective of fertility treatment.

To verify that the mRNA on the blots was intact in May, blots were probed with cDNAs to detect the high-molecular-weight root VSP (RN-T16) and β-amylase (pMSβA1); both of which gave the expected results of high expression in May when compared to December. At the present time we cannot explain the lack of expression of these P-transporter genes in May when P-demand by alfalfa would be relatively high, nor the high level of expression (with the expected pattern of fertility-induced effects) in December when little growth is occurring and soil temperatures are near 0 °C.

5. Summary and conclusions

Limited success has been forthcoming from efforts to genetically improve alfalfa forage yield. Selecting for greater disease resistance or improved winter survival has not resulted in unilateral improvement of alfalfa stand persistence. Our current understanding of the alfalfa genome is poor, and this will limit efforts aimed at improving alfalfa using genomic tools. Functional genomics represents an opportunity to improve yield and stress tolerance of alfalfa, but there is a dire need to identify genes that improve agronomic performance of alfalfa and to evaluate the function of these genes in the appropriate context (species, tissue, environment). Significant research effort aimed at gene identification and characterization, protein function, and their impact on phenotype is needed. Likelihood of success in efforts to improve agronomic performance of alfalfa will be enhanced by partnerships between public and private scientists.

References

Attwood, T., 2000. The babel of bioinformatics. Science 290, 471–473.

Avice, J.C., Ourry, A., Lemaire, G., Boucaud, J., 1996. Nitrogen and carbon flows estimated by ^{15}N and ^{13}C pulse-chase labeling during regrowth of alfalfa. Plant Physiol. 112, 281–290.

Barber, L.D., Joern, B.C., Volenec, J.J., Cunningham, S.M., 1996. Supplemental nitrogen effects on alfalfa regrowth and nitrogen remobilization from roots. Crop Sci. 36, 1217–1223.

Barnes, D.K., 1991. Minnesota fall dormancy and its relationship to winter injury. In: Proceedings of the 21st National Alfalfa Symposium, February 14–16, 1991, Rochester, MN. Cert. Alfalfa Seed Council, Inc., Davis, CA, pp. 56–60.

Barnes, D.K., Goplen, B.P., Baylor, J.E., 1988. Highlights in the USA and Canada. In: Hanson, A.A., Barnes, D.K., Hill Jr., R.R. (Eds.), Alfalfa and Alfalfa Improvement. Am. Soc. Agron. Monogr. 29, 1–24.

Beuselinck, P.R., Bouton, J.H., Lamp, W.O., Matches, A.G., McCaslin, M.H., Nelson, C.J., Rhodes, L.H., Sheaffer, C.C., Volenec, J.J., 1994. Improving legume persistence in forage crop systems. J. Prod. Agric. 7, 311–322.

Boyce, P.J., Volenec, J.J., 1992. β-Amylase from alfalfa taproots. Phytochemistry 31, 427–431.

Castonguay, Y., Nadeau, P., Lechasseur, P., Chouinard, L., 1995. Differential accumulation of carbohydrates in alfalfa cultivars of contrasting winter hardness. Crop Sci. 35, 509–516.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., Vad, K., 1993. Plant chitinases. Plant J. 3, 31–40.

Coutts, J.H., Kallenbach, R.L., Nelson, C.J., 2001. Determining plant density of alfalfa: to dig or not to dig? Agron. Abstr. C06-nelson094437-P. Amer. Soc. Agron., Madison, WI.

Cunningham, S.M., Volenec, J.J., 1996. Purification and characterization of alfalfa taproot vegetative storage proteins. J. Plant Physiol. 147, 625–632.

Cunningham, S.M., Volenec, J.J., 1998. Seasonal carbohydrate and nitrogen metabolism in roots of contrasting alfalfa (*Medicago sativa* L.) cultivars. J. Plant Physiol. 153, 220–225.

Cunningham, S.M., Volenec, J.J., Teuber, L.R., 1998. Plant survival and root and bud composition of alfalfa populations selected for contrasting fall dormancy. Crop Sci. 38, 962–969.

Cunningham, S.M., Gana, J.A., Volenec, J.J., Teuber, L.R., 2001. Winter hardiness, root physiology, and gene expression in successive fall dormancy selections from 'Mesilla' and 'CUF 101' alfalfa. Crop Sci. 41, 1091–1098.

Elgin Jr., J.H., Welty, R.E., Gilchrist, D.B., 1988. Breeding for disease and nematode resistance. In: Hanson, A.A., Barnes, D.K., Hill Jr., R.R. (Eds.), Alfalfa and Alfalfa Improvement. Am. Soc. Agron. Monogr. 29, 827–858.

Gana, J.A., Kalengamaliro, N.E., Cunningham, S.M., Volenec, J.J., 1998. Expression of β-amylase from alfalfa taproots. Plant Physiol. 118, 1495–1505.

Gana, J.A., Cunningham, S.M., Teuber, L.R., Volenec, J.J., submitted for publication. Genetic differences in winter hardiness are associated with expression of cold-acclimation responsive genes in *Medicago sativa* roots. Crop Sci.

Haagenson, D.M., Cunningham, S.M., Volenec, J.J., 2000. Autumn defoliation effects on cold acclimation, winter hardiness, and root gene expression of *Medicago sativa* L. Plant Physiol. Abstract #443. http://www.aspp.org/annual_meeting/pb-2000/2000.htm#.

Holin, F., 2001. Alfalfa's new crop. Hay Forage Grower 16, 30–37.

Holland, J.B., Bingham, E.T., 1994. Genetic improvement for yield and fertility of alfalfa cultivars representing different eras of breeding. Crop Sci. 34, 953–957.

Ipson, R.B., 1991. Thirty years of Wisconsin variety trials. M.Sc. Thesis. Department of Agronomy, University of Wisconsin, Madison, WI, 99 pp.

Levorson, J., Chlan, C.A., 1997. Plant chitinase consensus sequences. Plant Mol. Biol. Rep. 15, 122–133.

Michaud, R., Lehman, W.F., Rumbaugh, M.D., 1988. World distribution and historical development. In: Hanson, A.A., Barnes, D.K., Hill Jr., R.R. (Eds.), Alfalfa and Alfalfa Improvement. Am. Soc. Agron. Monogr. 29, 25–91.

Monroy, A.F., Castonguay, Y., Laberge, S., Sarhan, F., Vezina, L.-P., Dhindsa, R.S., 1993. A new cold-induced gene is associated with enhanced hardening at subzero temperatures. Plant Physiol. 102, 873–879.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.

Raghothama, K., 1999. Phosphate acquisition. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 665–693.

Salzer, P., Bonanomi, A., Beyer, K., Vogeli-Lange, R., Aeschbacher, R.A., Lange, J., Wiemken, A., Kim, D., Cook, D.R., Boller, T., 2000. Differential expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation, and pathogen infection. Mol. Plant-Microbe Interact. 13, 763–777.

Sheaffer, C.C., Lacefield, G.D., Marble, V.L., 1988. Cutting schedules and stands. In: Hanson, A.A., Barnes, D.K., Hill Jr., R.R. (Eds.), Alfalfa and Alfalfa Improvement. Am. Soc. Agron. Monogr. 29, 411–437.

Sheaffer, C.C., Barnes, D.K., Warnes, D.D., Lueschen, W.E., Ford, H.J., Swanson, D.R., 1992. Seeding-year cutting affects winter survival and its association with fall growth score in alfalfa. Crop Sci. 32, 225–231.

Smith, D., 1972. Cutting schedules and maintaining pure stands. In: Hanson, C.H. (Ed.), Alfalfa Science and Technology. Am. Soc. Agron. Monogr. 15, 481–496.