Quantitative Trait Locus Mapping of Winter Hardiness Metabolites in Autotetraploid Alfalfa (*M. sativa*)

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Abstract:

In winter hardy alfalfa cultivars, cold acclimation occurs prior to the onset of freezing temperatures and normally is accompanied with a series of metabolic and morphological adjustments. We are studying the accumulation pattern of metabolites throughout the autumn previous to freezing and relating them to winter survival in an F1 segregating population between the cross of *M. sativa* subsp. *sativa* and subsp. *falcata*. Morphological components and soluble carbohydrates, protein, amino-N groups, and free fatty acids were measured in 2001 and 2002 in the field. Broad sense heritability was intermediate for shoot and root mass and height, and for metabolites, ranged from low (TNC=0.04) to high (starch=0.80). The genetic correlation between winter injury was not significant for most of the metabolites, except for soluble protein and amino-N group concentrations. The presence of allele *a1* of *MSAIC B*, a cold-related gene, was positively associated with autumn plant height but negatively associated with root mass in the WISFAL-6 parent. Numerous QTL were detected for concentrations of metabolites. Our results suggest that winter injury and autumn biomass are controlled by different loci in this population.

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1. INTRODUCTION

Alfalfa originated in the Caucasus Mountains and surrounding regions from which it spread throughout much of Eurasia and North Africa, resulting in alfalfa germplasm with a wide diversity of winter hardiness. The most winter hardy alfalfa germplasm is *M. sativa* subsp. *falcata*, which grows primarily in colder and drier areas of Europe and Asia. Winter hardy alfalfa becomes dormant in response to shortening photoperiod and cooling temperatures typical of autumn, thus acclimating for the harsh environmental conditions of winter (McKenzie et al. 1988).

The accumulation of various compounds, including regulators of osmotic potential like sucrose and proline and of membrane fluidity like linoleic acid, takes place during hardening, helping cells to tolerate dehydration and maintain membrane integrity. The soluble carbohydrate concentration in alfalfa taproots during early autumn is not associated with cold tolerance, but winter hardy germplasm accumulates higher concentrations of soluble sugars in late autumn than do non-hardy cultivars (Cunningham et al. 1998; Alarcón-Zúñiga et al. 2001). Taproot starch concentrations may also be partially involved with winter hardiness as they can be converted to sugars when needed for acclimation (Boyce and Volenec, 1992). The accumulation of oligosaccharides, including raffinose and stachyose, has been linked to decreased winter injury (Cunningham et al. 2003). The concentrations of other compounds and the expression levels of a suite of genes has been shown to change under cold or winter stresses in alfalfa and many other plants (Thomashow, 1999).

Alfalfa cold acclimation is a complex phenomenon comprising a multitude of physiological and biochemical processes (Volenec et al. 2002). The objective of this paper is to provide a preliminary examination of the genetic relationships among concentrations of carbohydrates, soluble protein, amino-N groups, and fatty acids during late autumn, autumn plant growth, and winter injury using both quantitative genetic and molecular marker methods to detect quantitative trait loci (QTL).

2. RESEARCH METHODOLOGY

2.1 Experimental Population

Two genotypes, ABI408 (*Medicago sativa* subsp. *sativa*) and WISFAL-6 (*M. sativa* subsp. *falcata*) were crossed to form an F1 population of 200 individuals segregating for yield, winter injury, and autumn growth (Brummer et al. 2000). The 200 F1 individuals, two parents, and eight checks

were clonally propagated by stem cuttings in the greenhouse and transplanted at Ames, IA on June 1, 2001 and on May 15, 2002. The plot design in both years was a 14 by 15 quadruple alpha lattice design with 3 replications and 3 destructive harvest dates (August, November and April). Five plants per genotype were space planted at 16 cm apart within a plot in each replication with rows separated by 80 cm. The plots received no fertilization (P and K were above recommended levels for alfalfa cultivation) and were hand-weeded; insects were controlled chemically.

2.2 Growth Components, Winter Injury and Metabolite Analysis

Height was measured in late August and early November each year, after which the plants were dug, washed in water, and taproots, crowns, and shoots separated. The 3 tissues were frozen with liquid nitrogen and stored in dry ice for transportation to the lab, where they were kept at -80°C until freeze-drying. The freeze-dried samples were weighed and finely ground with a 1 mm mesh screen (UDY Cyclone, UDY Manufacturing, Fort Collins, Co). The following April, the 3rd block was scored for winter injury by the method of McCaslin and Woodward (1995), which included the digging of the complete plant and visual scoring on a scale of 1 = no injury, all plants symmetrical with equal shoot length to 5 = all dead plants.

The ground taproot samples were scanned by Near Infrared Reflectance Spectroscopy and reflectance measurements (log 1/R) between 1100 to 2500 nm, recorded at 4-nm intervals obtained with a scanning monochromator (NIRS Systems, Silver Springs, MD 20910). Fifty calibration samples, representing the range of H-values for the entire sample set, were selected for all the metabolites to analyze by wet chemistry.

Starch, sucrose, glucose and fructose were extracted using 80% ethanol and quantified by enzymatic assay (Sigma Chemical Co., St. Louis, MO; SCA20, sucrose; FA20, fructose; GAG020, glucose). Total non-structural carbohydrates (TNC) was estimated by means of the anthrone assay and scored using a glucose standard (Koehler, 1952). Residual extracted samples were dried at room temperature and the starch content determined by the alpha-amylase-amyloglucosidase-glucose and oxidase-peroxidase enzymatic assays (Sigma Chemical Co., St. Louis, MO; STA20). Soluble protein and amino-N groups were extracted with sodium phosphate and quantified by the BCA procedure (Smith et al., 1985). Total N concentration was obtained by dry combustion, and crude protein was estimated as total N x 6.25. Fatty acids were extracted with methanolic HCl and a C:17 internal standard was added to each sample. Fatty acids were esterified using 0.5% BHT in hexane (Sukhija and Palmquist, 1988) and quantified by gas chromatography. GC-

MS was used to verify the correct elution time of the free fatty acid methyl esters found by the GC (Roessner et al., 2001).

2.3 Statistical Analysis for Quantitative Traits and QTL Qapping

The analysis of variance of each trait was done in each harvest date and combining across years with all effects in the model considered random. Statistical analysis was performed by MIXED, CORR, and GLM procedures of Statistical Analysis System (SAS Institute, 1990; Little et al., 1996). Orthogonal contrasts were developed to determine differences among offspring and parents for each trait (Lynch and Walsh, 1998). The heritability on an entry mean-basis was estimated according to Holland et al. (2003):

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gl}^2}{l} + \frac{\sigma_e^2}{rl}}$$

where σ^2_g, σ^2_{gl}, and σ^2_e represent variance components due entry, entry x year, and experimental error, respectively; l is the number of years and r the number of replications. Standard error of heritability estimates was determined from variance components of the MIXED and IML procedures with entries as fixed effects (Falconer and Mackay, 1996; Lynch and Walsh, 1998). The genetic correlation for all the pairwise traits was determined from least square means for each entry in each environment. MANOVA was used to obtain SS of the cross product and determine genetic correlations (SAS Institute, 1990).

Single marker analysis was performed by the MIXED procedure to estimate association between QTL traits and marker loci with a probability level of $P<0.01$. The mean for each trait for individuals in the population for which the given marker was present (+) or abscent (-) were calculated to estimate the phenotypic effect of the marker allele. Markers were assigned to tentative linkage groups based on Robins et al. (2003).

3. RESULTS AND DISCUSSION

3.1 Quantitative Genetic Analysis

The metabolite concentration in the progeny was intermediate to the parents (Table 1), with no difference between the mean progeny and mean parental values (i.e., no mid-parent heterosis was present). Concentration of TNC, sucrose, glucose, soluble protein and amino-N groups were higher in ABI408 than WISFAL-6; for starch, linolenic acid, and total fatty acid

concentrations, WISFAL-6 was higher. The progeny showed transgressive segregation for all metabolites. The broad sense heritability on an entry mean basis ranged from low for TNC (0.07) to high for starch (0.80). Genetic correlations with winter injury in November across years were only significant for amino-N groups and soluble protein ($P<0.01$). The same low correlation was found between winter injury and metabolite content for the August harvest (data not shown); however, average metabolite content increased up to twofold from August to November, suggesting their importance for winter survival. Similar results have been found by Cunningham et al. (2003) and Dhont et al. (2003).

Table 1. Parental and progeny mean values, range of progeny values, broad sense heritability on a entry mean basis, and genetic correlations (WIr_A) with winter injury of metabolites measured on taproots in November 2001 and 2002 in Ames, IA and averaged across years

Trait	F1 Progeny vs parents		Progeny	Range		
	ABI408	Wisfal6	mean	Progeny	H^2	$WIr_A \pm SE$
TNC, mg g^{-1}DM	128**	104 **	111±14	70-155	0.04±0.1	-0.15±0.1
Starch, mg g^{-1}DM	255**	339	317±61	86-502	0.8±0.03	-0.19±0.1
Sucrose, mg g^{-1}DM	78*	62	67±23	18-141	0.5±0.07	0.07±0.1
Glucose, µg g^{-1}DM	1093	980	938±28	150-900	0.4±0.09	0.01±0.01
FAME, mg g^{-1}DM	13 **	16 *	15±1	12-18	0.5±0.08	0.20±0.1
Linoleic, mg g^{-1}DM	7.5	8.9 **	8±0.7	6-10	0.6±0.07	0.18±0.04
Sol. Protein, mg g^{-1}DM	60 **	54	55±4	26-68	0.5±0.07	0.26±0.1**
Amino-N, µmoles g^{-1}DM	122 **	92 *	104±31	40-191	0.5±0.08	0.44±0.1**

3.2 QTL Mapping for Fall Growth Components

We identified several QTL from each parent for all traits (Table 2). Marker locus *UGA769a1* had the strongest association with shoot mass and was also associated with root mass; its presence decreased both traits in the progeny. The presence of allele *a1* of *MSAIC B*, a cold-related gene (Laberge et al., 1993), was positively associated with autumn plant height but negatively associated with root mass in the WISFAL-6 parent. Importantly, little overlap between loci controlling autumn plant height or shoot mass was observed with those controlling winter injury. This is not surprising considering the low genetic correlation between these traits in this population (Brummer et al., 2000). These results raise the hope that biomass yield and winter injury can be manipulated independently, at least in some populations.

3.3 QTL Mapping of Metabolites

Numerous markers located in same linkage group (G) were associated with the concentration of the metabolites related to alfalfa winter hardiness that we measured (Table 3). Some loci were associated with multiple

metabolites. ABI408 carries alleles at two loci on G that have opposite effects, both of which explain about 11% of the phenotypic variation observed for this trait. Two markers associated with both linoleic and total fatty acid concentration (*ACG/CTG325* and *UGA328*) were also located in the linkage group G. Marker locus *UGA577* was strongly associated with soluble protein (R^2 = 11.1), although the presence of the marker allele decreased the soluble protein content. Few of these QTL correlate with those controlling the overall agronomically important phenotype of winter injury.

Table 2. Molecular markers associated with QTL for shoot and root biomass, plant height, and winter injury based on mean values across two years at Ames, IA. The parent carrying the marker allele is designated. The probability level, R^2, and phenotypic value for individuals with (+) or without (-) the marker allele are given.

LG	Closest marker	Parent	p-value	R^2	Mean (+)	Mean(-)
		Shoot mass, g				
B	*UGA161b2*	ABI408	0.0009	6.0	23.4	19.8
K	*AGC/CAC211*	ABI408	0.0083	3.7	23.7	21.5
L	*AGC/CAC352*	ABI408	0.0045	4.3	23.9	21.6
B	*UGA189a2*	Wisfal 6	0.0026	5.1	22.1	25.0
D	*UGA769a1*	Wisfal 6	0.0002	7.3	20.9	23.9
D	*bC3C-25aV2a1*	Wisfal 6	0.0015	5.3	23.8	21.2
G	*AGC/CAT452*	Wisfal 6	0.0048	4.9	23.4	20.6
I	*ACG/CTC168*	Wisfal 6	0.0073	3.8	23.7	20.6
M	*MS58a2*	Wisfal 6	0.0077	3.9	23.1	19.7
		Root mass, g				
B	*AGC/CTG247*	ABI408	0.0099	3.6	5.9	5.6
G	*UGA1208b*	ABI408	0.0064	4.1	5.5	5.9
J	*bC2A-9AV28b*	ABI408	0.0008	6.1	5.8	5.7
B	*ACG/CTA142*	Wisfal 6	0.0014	5.4	6.1	5.6
C	*MSAICAa1*	Wisfal 6	0.0077	3.8	5.9	5.6
D	*UGA769a1*	Wisfal 6	0.0005	6.4	5.5	5.9
J	*MSAIC Ba1*	Wisfal 6	0.0005	6.5	5.5	6.1
M	*MS58a2*	Wisfal 6	0.0004	7.0	5.9	5.1
		Autumn Plant Height, cm				
E	*ARC1H11b*	ABI408	0.0033	4.5	13.5	14.5
G	*UGA328b*	ABI408	0.0064	3.9	13.5	14.4
J	*UGA452b2*	ABI408	0.0040	4.7	14.5	13.5
A	*UGA36a4*	Wisfal 6	0.0011	6.1	13.3	14.5
B	*UGA85a3*	Wisfal 6	0.0095	3.8	14.5	13.6
G	*ACG/CTA301*	Wisfal 6	0.0022	4.9	13.7	14.8
J	*MSAIC Ba1*	Wisfal 6	0.000003	11.4	14.8	13.2
		Winter Injury, 1 = none to 5=dead				
B	*V25b1*	ABI408	0.00021	7.8	2.3	2.8
H	*AGC/CTT279*	ABI408	0.00361	4.6	2.4	2.2
J	*UGA191b2*	ABI408	0.00233	4.9	2.4	2.2
K	*UGA246b*	ABI408	0.00575	4.2	2.3	2.5
A	*UGA1208a1*	Wisfal 6	0.00989	3.6	2.2	2.4
D	*UGA452a2*	Wisfal 6	0.00008	8.5	2.2	2.4
D	*UGA769a2*	Wisfal 6	0.00845	3.9	2.4	2.2

Table 3. Molecular markers associated with QTL for soluble carbohydrates, soluble protein, amino-N groups and fatty acids based on mean values across two years at Ames, IA. The parent carrying the marker allele is designated. The probability level, R^2, and phenotypic value for individuals with (+) or without (-) the marker allele are given

LG	Closest marker	Parent	p-value	R^2	Mean (+)	Mean (-)
		Total Nonstructural Carbohydrates, mg g^{-1}DM				
M	*UGA122*	ABI408	0.0054	4.4	112	109
B	*ACG/CTG277*	Wisfal6	0.0010	5.7	112	112
D	*bC3C-25aV2*	Wisfal6	0.0013	5.4	111	112
K	*UGA328-2*	Wisfal6	0.0026	5.2	111	113
		Starch, mg g^{-1}DM				
E	*bN2-20aV55*	ABI408	0.0026	4.9	311	325
G	*UGA1208*	ABI408	0.000005	11.2	305	327
G	*UGA5*	ABI408	0.000007	11.2	331	309
N	*UGA906*	ABI408	0.0029	4.8	314	332
A	*Vg1H6*	Wisfal6	0.0003	7.8	326	308
J	*RC-1-51dT23V20*	Wisfal6	0.0039	4.5	309	323
		Sucrose, mg g^{-1}DM				
G	*UGA1208*	ABI408	0.0013	5.7	69	66
G	*Vg2D11-1/2*	ABI408	0.0032	4.8	67	66
D	*bC3C-25aV2*	Wisfal6	0.0034	4.5	66	69
D	*Hg2G1*	Wisfal6	0.0045	4.5	67	68
		Glucose, µg g^{-1}DM				
E	*UGA28*	ABI408	0.0004	6.9	1015	975
F	*AGC/CTC141*	ABI408	0.0031	4.7	975	1008
C	*V25-1*	Wisfal6	0.0042	5.1	1002	961
G	*AGC/CTT192*	Wisfal6	0.0007	6.1	986	1031
		Linoleic Acid, mg g^{-1}DM				
G	*ACG/CTG325*	ABI408	0.0011	5.6	8.2	7.9
G	*UGA328*	ABI408	0.0009	5.7	7.9	8.1
G	*UGA5*	ABI408	0.0022	5.4	7.9	8.1
A	*Vg1H6*	Wisfal6	0.0029	5.4	7.9	8.2
H	*UGA553*	Wisfal6	0.0092	3.8	8.1	7.8
		Total Fatty Acid Methyl Esters, mg g^{-1}DM				
G	*UGA328*	ABI408	0.0011	5.6	14.4	14.7
B	*RC2B-63BV8*	Wisfal6	0.0086	3.4	14.5	14.8
		Soluble Protein, mg g^{-1}DM				
E	*UGA577*	ABI408	0.000005	11.1	54	56
C	*AGC/CAT159*	Wisfal6	0.00063	6.2	56	54
		Amino-N groups, µmoles g^{-1}DM				
F	*UGA109-1*	ABI408	0.0042	4.8	106	102
D	*Hg2G1*	Wisfal6	0.0066	4.3	104	105
G	*AGC/CAA452*	Wisfal6	0.0033	4.8	104	104
J	*ACG/CAC324*	Wisfal6	0.0056	4.3	103	106

4. CONCLUSION

We have demonstrated that we can identify loci involved in important physiological and biochemical pathways underlying complex, agronomically important traits like winter injury. Further analysis will focus on identifying the precise regions of the genomes in which QTL for metabolite

concentration, biomass production, and winter injury reside, and on attempting to link the QTL to potential candidate genes.

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