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Development and commercialization of reduced lignin alfalfa

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Reducing lignin content in forage legumes can improve digestibility and, correspondingly, animal performance, and alfalfa (*Medicago sativa*) is the first genetically engineered crop commercialized for improved forage digestibility. Lignin reduction was achieved by downregulating the gene encoding caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT), and development of the commercial product, branded as HarvXtra, required the coordination of two research institutions and two companies, and more than 15 years of research and field trials. Lignin modification has positive impacts on forage management. Future developments will likely stack lignin modification with additional forage quality traits.

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Introduction

Because of its high nutritional quality, yield and adaptability, alfalfa (*Medicago sativa*) is the most cultivated forage legume in the world, with around 450 million tons grown on 30 million hectares worldwide, largely in the U. S. (30%), Europe (25%) and Argentina (23%) [1,2]. Alfalfa is a major source of protein in the livestock and dairy industries, and is cultivated for hay and silage, used fresh for grazing, or dehydrated to meal or pellets.

Genetically engineered (GE) glyphosate-resistant alfalfa was deregulated in the U.S. in 2010, and, since 2015, an alfalfa variety with reduced lignin (RL) has been available as a stacked trait with glyphosate resistance. Approximately 15% of the alfalfa currently grown in the U.S. is GE, and this is expected to grow to up to 50% within the next 10 years, when Canada and Mexico will probably also deregulate GE alfalfa for cultivation and import [3,4,5•]; GE alfalfa was de-regulated in Argentina in June 2018.

The last decade has seen an upsurge in research on the engineering of plant cell walls, mainly in the context of facilitating digestibility of lignocellulosic materials for biofuels [6–8]. Using modern molecular approaches, crops such as switchgrass [9] and sorghum [10], with potential dual uses for forage and bioenergy, are currently in various stages of pre-commercialization.

Here, we review experience from the development of RL alfalfa, from the discovery of the trait/gene in the laboratory to the development and release of the commercial product on the market.

Prior GE technology in alfalfa

Herbicide-resistant (HR) alfalfa was the fifth glyphosate-tolerant crop (and the first perennial) commercialized in the U.S. following canola, soybeans, cotton and corn, but has been the subject of legal controversies. After being deregulated and planted commercially in the US in 2005, it was regulated again in 2007 when a District Court ruled that risks of cross-contamination with non-GE alfalfa had not been sufficiently assessed by the USDA. The ban on new plantings was reversed in 2010 by the US Supreme Court and planting resumed in 2011 [11,12].

Development of glyphosate resistance in weeds is an increasing problem [13•] that will require multiple approaches to overcome in the future [14••]. However, farmers recognize glyphosate as the most effective broad spectrum herbicide currently available and feel satisfied with HR alfalfa, their main criticism being the elevated price of the seed [15••]. Forage yield, quality and feed safety are similar in HR and non-GE alfalfa [16].

Development of reduced lignin alfalfa

Natural mutations in lignin pathway genes (the so-called brown mid-rib mutants) confer enhanced cell wall digestibility; the engineered reduced lignin trait was first reported in caffeic acid/5-hydroxyferulic acid 3-*O*-methyltransferase (COMT)-suppressed tobacco plants [17••]. To develop RL alfalfa, a team of 12 scientists from the Samuel Roberts Noble Foundation and the U.S. Dairy and Forage Research Center, and Forage Genetics International (FGI), started a collaboration in 2000 under the name of the Consortium for Alfalfa Improvement (CAI). CAI was joined by the company Pioneer in 2003. Initial

studies evaluated individual gene knockdowns in the lignin biosynthetic pathway for effects on alfalfa forage composition, fiber digestibility and agronomic performance. Knockdown of a caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT), under control of the phenylalanine ammonia-lyase (PAL)2 promoter from *Phaseolus vulgaris* [18] for vascular tissue-specific expression, gave the desired lignin reduction [19••] and improvement in forage quality [20••], without any negative impact on forage yield or lodging scores (Table 1). This discovery, which was verified in further digestibility trials [21–23], represented the first step in the development and commercialization of RL alfalfa (Figure 1a).

The reduced lignin event which was used to develop commercial HarvXtra alfalfa varieties was generated via standard *Agrobacterium tumefaciens*-mediated transformation of alfalfa clone R2336 [24,25], using a binary vector containing two T-DNAs. The first harbored a CCoAOMT suppression cassette configured in an inverted repeat [26], under control of the *P. vulgaris* PAL2 promoter [18] and the 3′UTR sequence from nopaline synthase (NOS). The second T-DNA contained an nptII cassette regulated by the CaMV35S promoter and NOS 3′UTR [26]. After kanamycin selection, 1042 independent T0 generation events were transferred to soil for further assessment (Figure 1b), from which 389 were confirmed to possess the CCoAOMT suppression cassette. These were crossed with Ms208, a conventional male sterile plant to produce F1 populations tracing to each event. PCR screening identified small populations of marker free plants representing 74 independent

Table 1

Biochemical, agronomic and forage quality traits of RL alfalfa in the three different stages of development of the commercial product HarvXtra alfalfa

Discovery [18,19••,20••,21,22]	CCoAOMT activity	COMT activity	Klason lignin	S/G	NDF (% DW)	ADF (% DW)	ADL (% DW)	IVTD (% DW)
CCoAOMT RNAi	4.5*	150*	86*	0.87*	30.5	23.6	3.0*	87.3*
COMT RNAi	99.5	10.5*	79*	0.02*	32.7	25.9	3.7	86.3
CTRL	100	100	100	0.52	31.3	24.8	4	84.6

Research and development [26]	H-units	G-units	S-units	Total lignin	S/G	Lodging (0–9) *MS1*	Plant height (in.) *MS1*	Forage yield (t/ha) *MS1*
'KK179' event	4.2	53.7*	42.1*	129.1*	0.82*	6.0	5.8	9.92
CTRL	3.1	61.7	35.2	138.1	0.60	5.7	6.0	9.96

Commercial events *MS2* [31•,32,33]	2015				2016		
	Loc. 1	Loc. 2	Loc. 3	Loc. 4	Loc. 1	Loc. 3	Loc. 4
	Yield (t/ha)						
HarvXtra	7.0	5.8	6.9	8.6	15.0	16.2	20.1
CTRL	7.6	6.8	7.3	8.9	15.9	16.6	20.6
	Crude protein (g/kg)						
HarvXtra	230	218	226	234	187	186	182
CTRL	223	213	224	228	179	184	177
	NDF (g/kg)						
HarvXtra	387	388	351	351	441	403	416
CTRL	402	397	354	361	455	409	424
	ADL (g/kg)						
HarvXtra	74*	74*	67	65*	81*	79*	77
CTRL	84	82	72	73	88	85	82
	NDFD (g/kg)						
HarvXtra	443*	391*	447	453*	333*	333*	339*
CTRL	401	359	431	419	283	302	306
	RFQ [32]						
HarvXtra	297*	284*					
CTRL	252	246					

CCoAOMT, caffeoyl-CoA *O*-methyltransferase; COMT, caffeic acid *O*-methyltransferase; S/G, syringyl (S)/guaiacyl (G) monomer ratio; NDF, Neutral Detergent Fiber; ADF, Acid Detergent Fiber; ADL, Acid Detergent Lignin; IVTD, *In Vitro* True Digestibility; DW, dry weight; NDFD, Neutral Detergent Fiber digestibility; RFQ, Relative Feed Quality; Loc., Location. *MS1*: multi-site trials 2010–2012; *MS2*: multi-site trials 2015–2016. Enzyme activities and lignin amounts in "Discovery" are relative to the control (set at 100). H (hydroxyphenyl), G and S units of lignin are % of total units. Total lignin is expressed as mg/g dry weight.

* Significantly different from controls $P < 0.05$, unpaired two-sided *t*-test. In the discovery phase, CTRL was an empty vector control, in the development phase CTRL is C_0-Syn1, a near isogenic conventional control. In the commercialization phase, CTRL is the average of three reference alfalfa cultivars (54R02, DKA43-22RR, WL 355.RR) obtained from [30••].