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Brown-midrib genes in maize and their efficiency in dairy cow feeding. Perspectives for breeding improved silage maize targeting gene modifications in the monolignol and *p*-hydroxycinnamate pathways

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Abstract

Maize silage is widely used and comprises the largest part of roughage in dairy cows diets. However, the biological conversion of cell wall carbohydrates into fermentable sugars by rumen microorganisms is hindered by their embedding with lignins, as well as by *p*-hydroxycinnamic acid cross-linkages between wall components. The use of mutants inducing high cell wall digestibility, such as brown-midrib mutants and especially *bm3*, is a relevant alternative strategy in breeding silage maize for feeding value, somehow easier than stacking numerous favorable genomic locations with weaker effects. To date, six brown-midrib mutations have been described, but feeding value experiments with dairy cattle have mostly involved the *bm3* mutation, which had appeared until now the most promising. A synthesis of 36 published articles on dairy cow feeding with regular and *bm3* maize silages, allowing 43 and 42 comparisons for intake and milk production, respectively, highlighted an average extra intake by 1.16 kg per cow per day and an average extra milk production by 1.26 kg per cow per day for cows fed diets based on *bm3* silages. The primary benefit of the *bm3* mutation in dairy cattle feeding is thus an increased silage intake, allowing lower giving of costly concentrates in animal diets. In addition to genes inducing the brown-midrib phenotype, including the recently identified *bm5* and *bm6* mutations, a survey of genes involved in secondary wall assembly pointed out several putative targets to be considered in the monolignol and ferulate pathways, as well as several of their upstream regulators. Searches for mutants can be based on the transposon-tagging strategy, but also through gene deregulation, and especially gene editing with the development of the CRISPR/Cas9 technologies. The greater understanding of cell assembly and secondary wall lignification in maize together with the identification of a set of key-genes and their spatio-temporal regulations will allow moving from a limited number of brown-midrib mutants to a larger panel of genetic resources for silage maize improvement, with reduced negative side-effects.

Keywords: *maize, brown-midrib, cell wall, digestibility, dairy cow, intake, lignin, monolignol, ferulate*

Introduction

Maize is the most widely used forage crop in dairy cow feeding, and maize silage often comprises the largest part of roughage in dairy cows diets. Maize is a popular forage crop because it is easy to grow and high yielding, and because its silage is easy to preserve, very palatable, with high intake in animals, and high energy content. Maize is well adapted to mechanization from planting to harvest, but also for animal feeding.

The end of milk quotas in the European Union (EU) has increased the competition between milk production systems, with more or less intensive and rearing conditions, and between countries having variable social and environmental legislations, thus inducing variable production costs. In the EU, 58% of milk production takes place in only four countries (Germany 22%, France 17%, United-Kingdom 10%, and Netherlands 9%), including also in these latter countries significant variation for milk yield per cows, ranging from 8,700 in the Netherlands to 6,500 kg per

cow per year in France. Moreover, only 18% of dairy cows belong to herds with more than 100 animals in France, while it is nearly 45% in Germany and the Netherlands, and 75% in the United-Kingdom. The most intensive and efficient system is currently the one of Denmark, with cows yielding an average 9,800 kg per cow per year and 87% of herds comprising more than 100 cows (4% of EU milk production). Conversely, the less intensive ones are still those of Eastern Europe, but 7% of EU milk is nevertheless produced in Poland with animal yielding an average 3,400 kg per cow per year. In July 2016, milk was bought 270 € t⁻¹ to French producers, but 315 € t⁻¹ in early 2017, this latter price being nevertheless lower than production costs.

Facing an excess of milk production in the EU, the reduction of production costs in dairy farming is an inescapable requirement, whatever all regulatory conditions. In France, the higher production expenses are related to labor costs (25%), followed by mechanization and feeding costs (23 and 21%, re-

spectively). Lowering mechanization expenses have surely to be considered. However, feeding costs are a privileged target for expense reduction, as new feeding approaches can be managed in short term strategies. The use of grazing in dairy cow feeding can contribute to lowering corresponding costs, but meadow growth only occurs in spring and autumn. Moreover, grazing management is all the more complex as herds are greater. Maize silage is thus widely used for cattle feeding during winter and summer seasons, but also as a complementary resource with high energy content in cow diets during year more or long periods.

Maize breeding for silage use first focuses on yield, standability, and biotic and abiotic stress tolerance. Energy content, estimated as whole plant or cell wall (*in vitro*) digestibility is most often a secondary breeding target, taken into account when all other traits have been considered. As a consequence, while huge improvements have been achieved for other traits during the last fifty years, only limited increases have been observed for cell wall digestibility or energy value, even with a (little) decline in some cases. In addition to variable selection pressures between agronomy and feeding traits, this situation also results from partial antagonisms between these traits. A higher stover digestibility is related to reduced lignification and cross-linkages in the cell wall, with possible detrimental effects on agronomic traits, including disease and pest tolerance.

A synthesis of *in vivo* cell wall digestibility and energy values of maize hybrids registered between 1958 and 2002 has thus shown the decrease of cell digestibility across periods of breeding (Barrière et al, 2004a; Barrière et al, 2005). The NDFD (*in vivo* Neutral Detergent Fiber Digestibility) of early and medium early hybrids registered in France between 1958 and 1980 was equal to 51.1%, while it declined down to 45.7% in investigated hybrids registered between 1999 and 2002. The corresponding UFL (French energy forage unit for milk production, 1 UFL = 7.11 MJ) values decreased from 0.92 UFL to 0.87 UFL in hybrids registered in the two corresponding periods (complementarily, means UFL values were equal to 0.91, 0.89, and 0.88 in the 1981 - 1988, 1989 - 1993, and 1994 - 1998 periods, respectively). However, the whole dry-matter (DM) yield that was equal to 12.5 t ha⁻¹ for hybrids registered during the 1958-1980 period reached 18.1 t ha⁻¹ in hybrids registered during the 1999 - 2002 period. No very significant changes in whole plant grain content (average value 44.5%) were observed, but together with yield improvement, there were simultaneously major improvements in resistance to stalk lodging and stalk rotting, and tolerance to biotic and abiotic stresses.

As a consequence, changes in French registration rules of forage maize were set up since the end of the 1990 years. In addition to whole plant yield and standability criteria, newly registered hybrids must

satisfy to a threshold in UFL value which is calculated through a regression based on whole plant enzymatic solubility («M4 model», Dardenne et al, 1993). The decrease in maize hybrid energy value has thus been stopped, and high yielding modern hybrids have currently an UFL value close to the one of hybrids of the 1980 - 1990 years, nearing 0.89 - 0.90 UFL, still lower of the one of old hybrids that greatly contributed to the silage maize development, such as Inra258, LG11, Inra240, or Fronica (UFL values equal to 0.94, 0.91, 0.94, and 0.91, hybrids registered in 1958, 1970, 1972, and 1974, respectively). A similar genetic drift towards lower energy values was observed in northern European countries, and changes of registration rules since 1986 in the Netherlands allowed recovering modern hybrids with energy values nearly equal to the one of LG11.

Investigations with sheep in digestibility crates (Barrière et al, 2004a) have shown that silage maize energy value is first related to cell wall digestibility ($r^2 = 0.59$) and secondly to grain content ($r^2 = 0.29$), but not to cell wall content ($r^2 = 0.05$). Moreover, starch content higher than 30% in the diet can induce rumen acidosis with negative effects on cow health and milk production. In addition to agronomy traits, the main target in silage breeding is thus cell wall digestibility. Several QTL analyses have established that cell wall digestibility is a highly multifactorial trait, primarily related to lignin content, lignin structure, and ferulate cross-linkages. These biochemical and physical characteristics are governed by complex cascades of regulation during the secondary wall assembly and its lignification. Breeding for improved energy value is also made more complex by the fact that lignins and cross-linkages provide stiffness to tissues and vessels, contributing to plant standability, water and nutrient conduction, and biotic and abiotic stress tolerance. A possible breeding strategy is then to investigate mutant or deregulated genes for which significant induced improvements of plant cell wall digestibility have been proved or are expected, and secondly to breed the introgression of the more efficient ones in genetic backgrounds minimizing their possible negative effects on other agronomic traits. In maize and other grasses (most often C4 grasses), several brown-midrib mutations have been described and investigated, showing for some of them great potential in maize feeding value improvement. In addition, the role of numerous genes has been shown in the secondary cell wall assembly, which consequently can be considered as targets for mutation or deregulation in breeding program for improved feeding value of silage maize. After reviewing data illustrating the interest of brown-midrib mutants in maize breeding for improved feeding value, the similar interest of searching for mutations in genes involved at key positions in secondary wall biosynthesis and assembly will be presented and discussed

Maize brown-midrib mutations

Maize brown-midrib mutants exhibit a reddish-brown pigmentation of the leaf midrib and stalk pith, associated with lignified tissues, that becomes visible since the plants have about five expanded leaves (Figure 1). Leaf pigmentation fades as the plant matures but remains in the stalks. The first brown-midrib mutants were described and investigated successively by Kiesselbach (1922), Eyster (1926), and Jorgenson (1931). The latter established that these three brown-midrib mutations were «due to identical factors» and that the corresponding «brown-midrib character» segregated as «a simple Mendelian recessive» trait. This gene was later named *bm1*, when a second non allelic mutant was described by Burnham and Brinks (1932) which was named *bm2*. A little later, two other genes inducing the brown-midrib phenotype were described as *bm3* (Emerson, 1935) and *bm4* (Burnham, 1947). As the *bm1* gene, the *bm2*, *bm3*, and *bm4* genes originated from natural mutations and segregated as simple Mendelian recessive traits. No new maize brown-midrib mutations were characterized for nearly 60 years after the Burnham's paper (1947), despite the mention of additional natural brown-midrib mutants in the MaizeGDB database. Recent allelic tests of these latter mutants highlighted the three novel *bm5*, *bm6* and *bm7* loci (Haney et al, 2008; Ali et al, 2010). However, the *bm7* mutant was little latter shown to be allelic to *bm1* (Barrière et al, 2013).

Two brown-midrib mutations have been shown to alter genes of the monolignol pathway. The *bm1* mutation, assigned to maize chromosome 5 (bin 5.04), upstream the centromere (Jorgenson, 1931), affects the ZmCAD2 (Zm00001d015618, pos 101.49 Mbp) gene (Halpin et al, 1998). The *bm3* mutation, assigned to maize chromosome 4 (bin 4.05), upstream the centromere, affects the ZmCOMT (Zm00001d049541, pos 33.82 Mbp) gene (Vignols et al, 1995).

Two other brown-midrib mutations were shown to alter genes involved in metabolisms occurring upstream phenylpropanoid and monolignol pathways. The *bm2* mutation, located on maize chromosome 1 (bin 1.11), affect a methylenetetrahydrofolate reduc-



Figure 1 - Brown-midrib line F7803bm1

tase (MTHFR, Zm00001d034602, pos 297.61 Mbp) gene (Tang et al, 2014). The MTHFR gene encodes a protein which is involved in the methylation of homocysteine to generate methionine, thus allowing the subsequent production S-adenosyl-methionine (SAM), that is used as methyl donor for ZmCOMT (and other OMT) activity. The *bm4* mutation, located on chromosome 9 (bin 9.07), affects a folylpolyglutamate synthase (FPGS, Zm00001d048514, pos 157.62 Mbp) gene (Li et al, 2015), of which encoded protein acts upstream of the MTHFR and catalyzes the polyglutamylation of tetrahydrofolate (THF). It has indeed been shown that polyglutamylated folates are in plants the preferred substrates of folate-dependent enzymes.

The *bm6* mutation is not fully characterized. However, this mutation has been mapped to a 180 kb region of bin 2.01, in which ten genes were present (Chen et al, 2012). Among these ten putative candidates, only four have Arabidopsis orthologs significantly expressed in stems (genecat.mpg.de database), comprising a CCCH zinc finger (Zm00001d001952, pos 3.43 Mbp), a PHD finger-like (Zm00001d001951, pos 3.42 Mbp), an ubiquitin-like (Zm00001d001949, pos 3.42 Mbp), and IQ calmodulin (Zm00001d001953, pos 3.44 Mbp) encoding genes. Proteins of these four families play important roles in the regulation of many processes. More especially, several CCCH zinc finger genes have also been shown to colocalize with lignin and/or cell wall degradability QTLs in RIL progenies of Arabidopsis (Chavigneau et al, 2012) and maize (Barrière et al, 2016). In addition, three genes (GRMZM2G046852, GRMZM2G511859, and GRMZM2G342107) for which no functional annotation was available in Chen et al (2012) are no longer present in the B73 AGPv4 assembly.

The gene (or determinant) underlying the *bm5* mutation is still unknown. This mutation has been mapped in bin 5.04, near the centromere of which physical position is given between markers located at 89 and 140 Mbp (MaizeGDB database, IBM2 neighbors map). However, based on biochemical investigations, the *bm5* mutant plants exhibited traits corresponding to CCR deficiency hallmarks in their lignins (Méchin et al, 2014). It could thus be hypothesized that the *bm5* mutation could likely affect either a CCR enzyme also involved in monolignol biosynthesis or a (specific) upstream regulation factor of CCR enzymes. Complementarily, but less probably, the *bm5* mutation could affect another enzyme linked to the monolignol pathway and inducing similar lignin changes as those observed in CCR deficient plants. In maize, the ZmCCR1 (Zm00001d032152, bin 1.07, pos 214.57 Mbp) gene is considered to encode the enzyme primarily involved in the CCR activity during monolignol biosynthesis. Moreover, a brown coloration of midribs, husks, and stems was shown in RNAi down-regulated ZmCCR1 plants, with reduced lignin content (Park et al, 2012). It has been similarly shown that lignified tissues of CCR-deficient poplars or tobaccos

display an orange-brown coloration. However, other CCR and CCR-like/DFR proteins could also have a CCR activity, all the more as lignin content is only reduced in ZmCCR1 mutant or down-regulated plants (Tamasloukht et al, 2011; Park et al, 2012). Among the 12 ZmCCR1 closer paralog genes, only the CCR-like/DFR protein encoded by the ZmCCR3 gene (Zm00001d015513, bin 5.04, pos 94.71 Mbp) has the complete NWYCY motif which has been shown as essential for CCR activity (Lacombe et al, 1997; Escamilla-Treviño et al, 2010; Pan et al, 2014). In addition, both the ZmCCR1 and ZmCCR3 genes were shown significantly expressed in maize elongating internodes (Bosch et al, 2011). Conversely, the ZmCCR2 (Zm00001d019669, bin 7.02, pos 49.32 Mbp) gene is considered to be mostly involved in response to biotic and abiotic stresses and preferentially expressed in root. The ZmCCR3 gene could thus be considered as a possible candidate underlying the *bm5* mutation. However, if this hypothesis was the true one, it could also be assumed that it would already have been established. In addition, the ZmCCR3 gene, which is located in fairly close position of the ZmCAD2 gene (*bm1* mutation, pos 101.49 Mbp), is also quite close to the Zm4CL1 (Zm00001d015459, pos 91.46 Mbp) gene in bin 5.04, of which sorghum ortholog is disrupted in the sorghum brown-midrib *bmr2* mutation (Saballos et al, 2012). No CCR deficiency markers have been investigated in *bmr2* sorghum mutants (and are probably not expected).

Different mutation events could have occurred for each brown-midrib mutant. At least two different mutation events underlay the *bm3* mutants (Vignols et al, 1995; Morrow et al, 1997), both likely related to transposon activities. Five different events were shown underlying *bm1* mutants (Barrière et al, 2013). Two events correspond to insertions of two and four bases, disrupting the reading frame, respectively. One SNP in an area encoding a between-species conserved amino acid motif of the ZmCAD2 enzyme, and another SNP in an area encoding the conserved amino acid motif involved in the binding of the zinc ion at the catalytic site of the ZmCAD2 enzyme, were shown in two other *bm1* origins. Finally, an insertion of a (retro-) transposon element in the exon 2 was also shown for a recently described *bm1* origin. To date, no allelism variations have seemingly been investigated for the *bm2* and *bm4* brown-midrib mutations (and the *bm5* and *bm6* related genes are still unknown).

Effects of brown-midrib mutations on maize cell wall traits

The consequences of the *bm1* mutation on maize lignin content and structure were first described forty years after the description of the mutation (Kuc and Nelson, 1964; Gee et al, 1968). Mature maize *bm1* plants have a lignin content that is reduced by 10 to 20%, a slight decrease in ferulic acid esters and

substantially reduced contents (about 40%) in *p*-coumaric esters and ferulate ethers (Provan et al, 1997; Barrière et al, 2004b). The frequency of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) thioacidolysis monomers was similar in *bm1* and normal plants, showing that the *bm1* mutation does not specifically affect one of the lignin units. However, the reduced recovery of thioacidolysis monomers reveals that the frequency of lignin units involved only in β -O-4 bonds was about 50% lower in *bm1* plants than in lignins of normal plants, indicating that lignins of *bm1* plants were substantially enriched in carbon-carbon inter-unit linkages (Halpin et al, 1998; Barrière et al, 2004b). Lignins of *bm1* plants are also typified by a substantial incorporation of coniferaldehyde and, to a lower extent, of sinapaldehyde and *p*-hydroxybenzaldehyde into the polymer (Jacquet, 1997; Kim et al, 2002; Kim et al, 2003; Barrière et al, 2004b). This notable incorporation of *p*-hydroxy-cinnamaldehyde-derived compounds in *bm1* lignins was in agreement with mutations of the ZmCAD2 gene.

Most investigations on maize brown-midrib mutants, including those on cell wall biochemical traits, have focused on *bm3* plants, probably because the *bm3* mutation allows the greater improvements in cattle feeding value, at least before the discovery of *bm5* and *bm6* mutants of which *in vivo* feeding value is not yet investigated. Kuc and Nelson (1964) first established that the frequency of syringyl (S) units was heavily reduced in *bm3* lignified cell wall. They also suspected the occurrence of additional, but not yet identified units, that were shown later to be 5-hydroxy-guaiacyl units (Lapierre et al, 1988), involved in novel benzodioxane lignin structures (Ralph et al, 2001). Maize *bm3* plants had a lignin content reduced by 25% to 40%, but lignins in *bm3* mutant appear to inhibit proportionally more cell-wall digestibility than normal lignin (Thorstenon et al, 1992). This fact is likely related to a higher frequency of coniferaldehyde units in the polymer and more inter-unit linkages (Grabber et al, 1998). Despite the ZmCOMT gene, which encodes the enzyme primarily involved in the formation of S lignin units via methylation of 5-hydroxyconiferaldehyde, is completely deficient in *bm3* maize, S lignin units are heavily depleted, but not absent in *bm3* lignins. S units may therefore originate from another enzymatic activity, but not from CCoAOMT which have strict affinity for CoA esters. Lignins of *bm3* mutant plants also have fewer *p*-coumarate esters, a reduction consistent with the preferential acylation of S units by *p*-coumaric acid. In addition, *bm3* plants are not altered in their content of alkali-releasable ferulic acid (Barrière et al, 2004b).

Reduced lignin content by nearly 17%, nearly no change in *p*-coumarate and ferulate ester levels, and a significant decrease in ether ferulate levels were observed in *bm2* plants (Barrière et al, 2004b). In addition, the frequency of β -O-4-linked G units was reduced in *bm2* plants, whereas the frequency of β -

O-4-linked S units was not affected (Chabbert et al, 1994; Barrière et al, 2004b). A similar trend for lignin content was observed in *bm4* plants, with a reduced frequency of β -O-4-linked G units, no changes in *p*-coumarate and ester ferulate levels, and a tendency towards a reduction of ether ferulate levels. In addition, thioacidolysis of *bm2* and *bm4* lignins did not reveal any incorporation of unusual units, in contrast to *bm3* and *bm1* lignins. Lignins and *p*-hydroxycinnamates of *bm2* and *bm4* mutants thus have similarities that could possibly be consecutive of mutations located upstream the monolignol pathway. In addition, both *bm2* and *bm4* lines have poor vigor, at least after backcrossing in early germplasm, together with poor pollination.

Based on plants grown in glasshouse and with stems harvested at grain maturity, the lignin content in the cell wall of *bm5* mutants was reduced by nearly 15% (Méchin et al, 2014). The *bm5* mutation was also found to significantly reduce the level of *p*-coumarate esters by nearly 25% while a higher level of measurable ferulate was shown in *bm5* plants, that could originate either from a higher level of ferulic acid linked to arabinoxylans and/or from the reduced lignin content of *bm5* plants. In addition, lignins of *bm5* plants have i) an increased frequency of resistant inter-unit bonds mainly involving guaiacyl (G) units, ii) an increased frequency of free-phenolic G units in lignins and iii) the release after thioacidolysis of the

«A_G» 1,2,2-trithioethylguaiacol marker in unusually high levels, indicating the incorporation of free ferulic acid into lignins (Méchin et al, 2014), all traits previously observed in plants with CCR deficiency. The enzymatic cell wall digestibility of *bm5* plant stems was found very significantly improved (INRA Lusignan unpublished data). In *bm6* plants cropped and harvested in similar conditions as *bm5* ones, lignin content was only reduced by 9%, but cell wall digestibility was nevertheless significantly improved (INRA Lusignan unpublished data).

Effects of brown-midrib mutations on cell wall related gene expression

Only a few investigations have seemingly been devoted to comparative gene expression in brown-midrib maize plants, with data available for only a not exhaustive subset of genes involved in monolignol biosynthesis and polymerization (Guillaumie et al, 2007; Guillaumie et al, 2008). As expected, the ZmCAD2 and ZmCOMT genes are very significantly under-expressed in *bm1* and *bm3* mutant lines, respectively (Table 1). The ZmSAMS1, involved in the methyl donor pathway for OMT enzymes, as well as the SBP1 ZRP4-like OMT of which role is not established, are over-expressed in *bm3* plants. This latter OMT could contribute to syringyl alcohol biosynthesis, as it was shown that S units were only reduced to

Table 1 - Comparative expression in brown-midrib young plants of main genes involved in monolignol biosynthesis and polymerization.

Gene	Gene name	chr-pos	F2bm1/F2	F2bm2/F2	F2bm3/F2	F2bm4/F2
ZmPAL	Zm00001d017274	5-191.41	<i>1.61</i>	3.66	0.93	4.06
ZmC4H1	Zm00001d009858	8-85.45	-	-	-	-
Zm4CL1	Zm00001d015459	5-91.46	<i>0.89</i>	<i>1.20</i>	<i>1.77</i>	<i>3.22</i>
ZmHCT1	Zm00001d017186	5-188.27	<i>1.05</i>	<i>1.19</i>	<i>1.72</i>	<i>2.01</i>
ZmC3H1	Zm00001d043174	3-190.63	-	-	-	-
ZmC3H2	Zm00001d038555	6-159.79	-	-	-	-
ZmCCoAOMT1	Zm00001d036293	6-82.19	<i>0.50</i>	<i>0.74</i>	<i>1.23</i>	<i>1.52</i>
ZmCCoAOMT2	Zm00001d045206	9-16.08	-	-	-	-
ZmCCR1	Zm00001d032152	1-214.57	-	-	-	-
ZmF5H1	Zm00001d032468	1-227.65	-	-	-	-
ZmCOMT	Zm00001d049541	4-33.82	<i>0.66</i>	<i>1.43</i>	0.17	<i>1.60</i>
ZmCAD2	Zm00001d015618	5-101.49	0.38	<i>1.36</i>	<i>2.02</i>	<i>2.82</i>
ZRP4-SBP1-OMT	Zm00001d004689	2-131.24	<i>0.77</i>	<i>1.58</i>	<i>2.28</i>	<i>3.95</i>
ZRP4-OMT	Zm00001d029359	1-67.31	<i>0.80</i>	<i>2.54</i>	<i>1.10</i>	<i>3.27</i>
ZmSAMS1	Zm00001d040697	3-58.92	<i>0.63</i>	<i>1.68</i>	<i>2.42</i>	<i>2.63</i>
Laccase	Zm00001d042906	3-183.58	0.34	0.28	<i>1.00</i>	<i>0.55</i>

PAL - Phenylalanine/tyrosine ammonia lyase, C4H - Cinnamate-4-hydroxylase, 4CL - 4-Coumarate-CoA ligase, HCT - Hydroxycinnamoyl-CoA shikimate/quininate hydroxycinnamoyl transferase, C3H - *p*-Coumaroyl-shikimate/quininate 3-hydroxylase, CCoAOMT - Caffeoyl-CoA O-methyltransferase, CCR - Cinnamoyl-CoA reductase, F5H - Ferulate-5-hydroxylase, COMT - Caffeic acid O-methyltransferase, CAD - Cinnamyl alcohol dehydrogenase, SAMS - S-adenosyl-methionine synthetase, ZRP4 - Zea root preferential, SBP1 - Saffener binding protein1. Gene chromosome and position as Mbp in the maize B73 reference genome, genome assembly AGPv4; italicized ratio indicated that brown-midrib and regular F2 values were not significantly different; - = not available data, data from Guillaumie et al, 2007.

nearly 40% of regular lignins in *bm3* lignins (Barrière et al, 2004b). In Arabidopsis, a species without maize ZRP4-like OMT orthologs (even if definite conclusion cannot obviously be drawn), S lignins are almost missing in the AtOMT1 mutant (Goujon et al, 2003). The ZmPAL gene was highly over-expressed in *bm2* and *bm4* plants, which both are affected upstream this entry gene of the monolignol pathway. Based on this subset of genes, the *bm4* mutation induced the most numerous gene deregulations with over-expression of Zm4CL1, ZmHCT1, ZmCAD2, two ZRP4-OMT, and ZmSAMS1 genes. In addition, one Atlac17 ortholog laccase was shown under-expressed in *bm1* and *bm2* plants. In Arabidopsis, Atlac17, with Atlac4, has been shown to significantly contribute to the constitutive lignification of floral stems (Berthet et al, 2011). Similarly, Bdlac5, a Brachypodium Atlac17 ortholog, has also been shown significantly involved in monolignol polymerization (Wang et al, 2015). Several Arabidopsis Atlac17 orthologs are present in the maize genome, but their respective roles in monolignol polymerization are still unknown, with possibly differential spatio-temporal regulations.

Feeding value of maize *bm3* hybrids based on measurements with sheep in digestibility crates

The effect of maize brown-midrib mutations on feeding value was first evidenced by Barnes et al (1971). Since this date, many studies were made with brown-midrib plants, and most often with *bm3* plants, which proved very early to be powerful mod-

els in investigating variations in maize cell wall digestibility and feeding value in cattle, most often dairy cattle. Because comparison of large number of genotypes is not possible in experiments with dairy cattle, a study of cell wall digestibility in regular and *bm3* isogenic hybrids has then been done based on measurements with wethers in digestibility crates (Barrière et al, 2004a). Considering 31 maize hybrids (and 87 measurements) including 24 registered hybrids between 1958 and 1993 and 7 experimental hybrids, the improvement of cell wall digestibility in their isogenic *bm3* counterparts ranged from 0.9 to 15.8 percentage points, with an average improvement equal to 8.6 percentage points (average NDFD values equal to 49.7% and 58.3% in regular and *bm3* hybrids, respectively). The average grain content of *bm3* hybrids was only reduced by 1 percentage point (42.2% and 41.2%, respectively), while the average whole plant dry-matter yield was reduced by nearly 2 t ha⁻¹, from 14.9 to 12.9 t ha⁻¹ in regular and *bm3* hybrids. There was also a tendency to a greater efficiency of the *bm3* mutation for cell wall digestibility improvement when corresponding regular hybrids were of lower cell digestibility (Table 2). Energy value improvements were limited in *bm3* isogenic hybrids of Inra258 and Inra240, which are both of high energy value in their regular forms. However, improvement was great in Dk265*bm3*, even if the Dk265 regular hybrid is also of high energy value.

Breeding lines in *bm3* background is illustrated by the two INRA Lusignan lines F7026*bm3* (lodent and F113*bm3* related, released in 1996) and F7067*bm3* (BSSS and F7026*bm3* related, released in 2003). The latter line allowed producing experimental hybrids

Table 2 - Comparison of normal and *bm3* hybrids for digestibility and agronomic traits.

	OMD(%)	NDFD (%)	Yield (t ha ⁻¹)	UFL (/kg DM)	Grain (%)
Inra258 (registered in 1958)	72.9	55.0	12.0	0.94	42.0
Inra258 <i>bm3</i>	74.5	60.6	11.3	0.97	44.7
LG11 (registered in 1970)	71.0	50.2	12.5	0.91	43.7
LG11 <i>bm3</i>	74.0	60.4	11.5	0.96	43.3
Inra240 (registered in 1972)	72.6	56.1	12.3	0.94	48.2
Inra240 <i>bm3</i>	74.8	57.0	11.9	0.98	42.6
Puma (registered in 1982)	71.1	48.8	12.5	0.91	46.1
Pumab <i>bm3</i>	73.0	54.1	11.5	0.94	41.3
Dekalb265 (registered in 1987)	71.8	51.4	13.8	0.92	45.0
Dekalb265 <i>bm3</i>	75.8	62.1	12.5	0.99	42.3
Rh162 (registered in 1990)	67.6	43.4	17.1	0.85	43.6
Rh162 <i>bm3</i>	72.6	55.8	14.4	0.93	40.9
Rh185 (registered in 1994)	70.9	48.7	15.2	0.91	49.0
Rh185 <i>bm3</i>	73.7	57.4	11.0	0.95	48.3
F618 x F271	68.2	47.0	17.1	0.85	42.1
F618 <i>bm3</i> x F271 <i>bm3</i>	72.6	57.0	14.8	0.93	39.3
F7026 <i>bm3</i> x F2 <i>bm3</i>	73.6	59.4	12.5	0.95	45.3
F7067 <i>bm3</i> x F2 <i>bm3</i>	73.8	58.8	14.4	0.96	47.8
(F7 x F2) x B52	66.3	46.9	17.4	0.74	29.3
(F7 <i>bm3</i> x F2 <i>bm3</i>) x B52 <i>bm3</i>	71.1	55.3	12.8	0.93	28.1
H28 x W401	67.1	47.8	18.7	0.79	32.8
H28 <i>bm3</i> x W401 <i>bm3</i>	72.5	63.6	16.5	0.92	31.5

OMD - *in vivo* organic matter digestibility, NDFD - *in vivo* NDF digestibility with NDF neutral detergent fiber, UFL - French forage energy value for milk production, with 1 UFL = 7.11 MJ (INRA Lusignan unpublished data).

Table 3 - Comparison of MBS847 x F2 normal and *bm3* related hybrids for digestibility and agronomic traits.

	OMD(%)	NDFD (%)	Yield (t ha ⁻¹)	UFL (/kg DM)	Grain (%)
MBS847 x F2 (or Adonis)	70.4	48.7	16.2	0.90	45.5
MBS847 <i>bm3</i> x F2 <i>bm3</i>	73.9	56.2	13.5	0.97	42.2
(MBS847 x W117) x F2	73.7	53.9	15.3	0.94	46.2
(MBS847 <i>bm3</i> x W117 <i>bm3</i>) x F2 <i>bm3</i>	73.9	60.7	13.0	1.01	40.8

legend identical to the one of [Table 2](#) (INRA Lusignan unpublished data).

of which whole plant yield fitted well with the average silage production in most of French dairy cow rearing, and also corresponded with water resource availabilities. In addition, *bm3* hybrids are similarly of great interest for late maize germplasm of which cell wall digestibility is also often lower than the one of earlier genotypes, a fact that could also contribute to explain a greater development of *bm3* hybrids in the USA than in (northern) Europe markets. High cell wall digestibility and energy value improvements were thus shown in the *bm3* isogenics of the (F7 x F2) x B52 and H28 x W401 (medium) late hybrids, of which regular forms are of low cell wall digestibility and energy value, with however a great yield decrease in the *bm3* form of the (F7 x F2) x B52 hybrid. The low cell wall digestibility of the B52 line is also related to the great tissue stiffness of this line which is a well-known resource for European corn borer tolerance.

The efficiency of the *bm3* mutation in the improvement of cell wall digestibility was also highlighted through a comparison of hybrids derived from the regular MBS847 x F2 hybrid, which was registered in France in 1984 as Adonis ([Table 3](#)). The corresponding *bm3* isogenic hybrid has an *in vivo* cell digestibility (NDFD) increased by nearly 8 percent points and an energy value increased by 0.07 UFL kg⁻¹ DM. An intermediate improvement was obtained in the regular three-way hybrid including the Wisconsin line W117, which has been shown to be a parent giving progenies with significantly improved feeding values. An unexpected result was related to the still improved and very high feeding value of the *bm3* three-way hybrid, which reached an energy value equal to 1.01 UFL that is the one of barley grains. This also leads to suspect that the quality traits of W117 are not only related to lignin content, but likely also to differences in lignin structures, in type and frequency of cell wall cross-linkages, or possibly in still other unknown traits.

Feeding value of *bm3* hybrids based on experiments with dairy cattle

The (possible) improvement in performances of cattle fed brown-midrib maize plants was quite only investigated with the maize *bm3* mutant, because, compared to other maize brown-midrib mutants, the maize *bm3* mutant appeared to be especially improved in cell wall digestibility, at a greater extent than the *bm1* mutant, and because the agronomic traits are much depressed in *bm2* and *bm4* plants.

Most of the studies with *bm3* maize silage have been conducted on dairy cows which are among the ruminant livestock the animals with the highest energy and nutrient requirements.

The first experiments with dairy cattle are likely those done by [Colenbrander et al \(1972, 1973\)](#) on heifers. They noted higher intake and body weight gain in animals fed *bm3* silage, compared with those fed regular genotypes. The first published comparison of regular and *bm3* maize silages fed to lactating cows is likely the one by [Frenchick et al \(1976\)](#), and then, 36 articles can be found in different journals from 1976 to 2017, most often in the *Journal of Dairy Science*. These articles gathered 44 comparisons of dairy cattle feeding with regular and *bm3* silages. However, no experiments were seemingly published during the 12 successive years between 1987 and 1998 ([Figure 2, Table 4](#)).

Whereas the higher efficiency of *bm3* silage maize for cattle feeding has been clearly established through the experiments with lactating cows published between 1976 and 1986, breeders were simultaneously disappointed by the reduced yield, and also the lodging susceptibility, sometimes lower disease and pest tolerance, and reduced tolerance to dry conditions of *bm3* hybrids. The recent and renewed interest in *bm3* hybrids for dairy cattle feeding is related to the great improvements in agronomic values of maize germplasm during the last 25 years, making possible to bred *bm3* hybrids with yield and biotic and abiotic stress tolerances, approaching that of regular hybrids. In addition, the genetic drift towards lower feeding values of the parental lines used in modern medium early, medium late, and late hy-

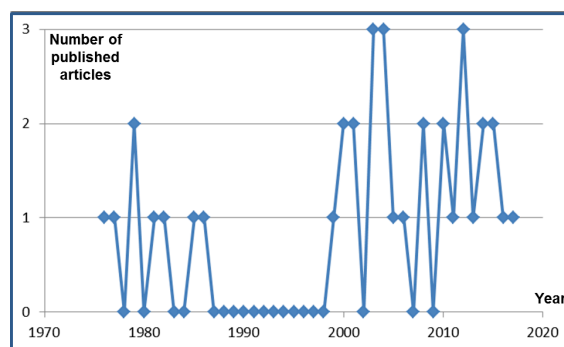


Figure 2 - Number of yearly published articles between 1976 and 2017 reporting comparisons of dairy cow performances when fed regular or *bm3* maize silages.

brids strengthened the interest of breeding for quality traits, including using strategies based on brown-midrib mutations, and especially *bm3*.

Data for intake, milk production, and body weight variations given in the 36 articles have been synthesized (Table 4), allowing 43 and 42 comparisons for intakes and milk productions, respectively, and 22 comparisons for weight average daily gain variations (ADG). According to experiments, diets were given to cows either as total mixed rations (TMR), or with separate giving of maize silage and concentrates (MZS), but total diet values are given in table 4. Milk productions were adjusted if necessary to 4% fat corrected milk (FCM) according to the formula $FCM4\% = [0.4 \times \text{milk} + (15 \times \text{milk} \times \text{fat})]$, with milk as kg and fat content

as percentage.

Average percentages of silage and total forage given in cow diets were the same in normal maize based diets (51% and 61%, respectively) and *bm3* maize based diets (52% and 61%, respectively). However, according to experiments, the proportion of silage in the diet, which were nearly similar within each experiment for normal and *bm3* hybrids, ranged between 30% and 85%.

Average intake was 1.16 kg per cow per day higher in cows fed *bm3* diets. Intakes of *bm3* diets were equal or lower than the one of normal diet only in five experiments with correlative lower milk production with the *bm3* diet in only one experiment comparing not isogenic hybrids. In 15 experiments (out of

Table 4 - Feeding efficiency of *bm3* maize silage in dairy cattle, from experiments published between 1976 and 2017.

Reference	Year	Hybrid	Silage % N/bm3	Forage % N/bm3	Intake N	Intake bm3 - N	FCM (4%) N	FCM bm3 - N	ADG bm3 - N
Frenchick et al	1976	isogenic	49/49	59/59	20.0 (TMR)	0.9	18.9	0.7	88
Rook et al	1977	isogenic	60/60	60/60	18.0 (MZS)	1.1	34.0	-0.1	14
Rook et al	1977	isogenic	85/85	85/85	17.6 (MZS)	2.6	22.0	0.7	42
Keith et al	1979	isogenic	60/60	60/60	21.6 (TMR)	0.4	24.3	0.9	na
Keith et al	1979	isogenic	75/75	75/75	21.4 (TMR)	0.6	24.5	0.8	na
Sommerfeldt et al	1979	isogenic	55/57	55/57	17.8 (MZS)	0.7	22.5	-0.7	106
Block et al	1981	isogenic	65/65	65/65	19.6 (TMR)	2.3	28.8	0.6	1244
Stallings et al	1982	isogenic	49/47	49/47	16.5 (MZS)	0.6	18.8	-0.6	80
Hoden et al	1985	isogenic	80/80	80/80	16.2 (MZS)	1.0	22.2	0.7	165
Hoden et al	1985	isogenic	78/86	78/86	17.2 (MZS)	1.7	21.6	1.7	115
Weller and Phipps	1986	isogenic	70/70	70/70	10.9 (MZS)	0.6	15.3	3.3	90
Oba and Allen	1999	isogenic	45/45	56/56	23.5 (TMR)	2.1	37.9	2.4	100
Bal et al	2000	not	32/40	47/60	28.4 (TMR)	0.0	38.6	0.5	40
Oba and Allen	2000	isogenic	32/36	40/44	23.9 (TMR)	0.8	32.9	1.2	300
Oba and Allen	2000	isogenic	51/56	64/69	21.5 (TMR)	1.4	33.1	3.0	20
Ballard et al	2001	not	31/31	50/50	na	na	33.1	2.2	na
Tine et al	2001	isogenic	60/60	60/60	22.8 (TMR)	2.4	31.0	2.0	170
Barrière et al	2003	isogenic	75/75	75/75	20.0 (MZS)	2.6	na	na	na
Moreira et al	2003	not	40/40	75/75	17.6 (TMR)	1.9	32.1	1.4	na
Qiu et al	2003	isogenic	41/41	51/51	25.0 (TMR)	1.2	33.5	0.6	na
Qiu et al	2003	isogenic	50/51	63/64	23.5 (TMR)	3.3	32.9	2.1	na
Barrière et al	2004c	isogenic	76/76	76/76	22.2 (MZS)	1.3	na	na	na
Cherney et al	2004	not	60/57	67/64	21.8 (TMR)	0.9	35.0	2.1	na
Cherney et al	2004	not	60/60	67/67	20.9 (TMR)	1.8	34.9	2.2	na
Ebling and Kung	2004	not	42/42	60/60	23.4 (TMR)	1.5	37.7	1.9	na
Taylor and Allen	2005	isogenic	38/38	48/48	23.6 (TMR)	1.6	39.0	2.3	200
Weiss and Wyatt	2006	not	55/55	55/55	25.0 (TMR)	0.2	36.6	-0.8	na
Gehman et al	2008	not	49/54	53/58	20.1 (TMR)	1.0	37.3	3.4	na
Kung et al	2008	not	45/45	55/55	26.9 (TMR)	-0.1	44.9	1.0	250
Castro et al	2010	not	40/40	56/56	24.7 (TMR)	1.3	35.7	0.7	210
Holt et al	2010	not	31/31	57/57	26.5 (TMR)	1.2	33.7	-2.0	na
Jung et al	2011	isogenic	41/42	52/52	31.4 (TMR)	0.1	44.4	2.5	na
Barlow et al	2012	not	36/36	52/52	22.4 (TMR)	0.6	32.3	3.0	na
Ramirez et al	2012	not	40/40	55/55	23.6 (TMR)	1.4	28.2	1.0	na
Stone et al	2012	not	40/42	56/58	18.1 (TMR)	2.0	45.7	2.5	na
Holt et al	2013	not	35/35	60/60	24.7 (TMR)	1.1	37.0	0.9	70
Akins and Shaver	2014	not	40/40	60/60	23.9 (TMR)	0.5	39.3	0.5	0
Gorniak et al	2014	not	50/50	50/50	22.5 (TMR)	-1.0	32.3	-1.3	0
Gorniak et al	2014	not	73/73	73/73	19.8 (TMR)	0.0	27.1	2.4	161
Ferraretto et al	2015b	not	43/42	66/65	26.4 (TMR)	1.7	47.0	1.0	0
Lim et al	2015	not	35/35	59/59	29.8 (TMR)	0.8	46.4	1.9	na
Lim et al	2015	not	35/50	59/59	29.8 (TMR)	0.0	46.1	1.5	na
Genero et al	2016	not	54/54	54/54	24.8 (TMR)	1.5	31.8	0.1	na
Hassanat et al	2017	not	59/59	65/65	25.8 (TMR)	1.6	34.1	2.5	na
mean	-	-	51/52	61/61	22.4	1.16	32.5	1.26	158
mean isogenic	-	-	59/60	63/64	20.7	1.40	27.5	1.27	195
mean not isogenic	-	-	45/46	58/59	24.0	0.90	36.4	1.26	146

comparisons between isogenic or not isogenic hybrids are noted isogenic and not, respectively; percentage of silage in the diet with normal and *bm3* hybrids (N/bm3); percentage of total forage in the diet with normal and *bm3* diets (N/bm3); intake as kg/day in normal diets, and intake differences between *bm3* and normal diets, diets given to cows either as total mixed ration (TMR) or separately for maize silage (MZS) and concentrates; fat corrected milk yield (4% FCM) in normal diets, and differences in milk yield between *bm3* and normal diets; average daily gain (ADG) differences between *bm3* and normal diets given as g per day; na are not available data

43), intakes of cow fed *bm3* diets were at least 1.5 kg per cow per day higher than those of cows fed normal silage diets. Average milk yield was 1.26 kg per cow per day higher in animals fed *bm3* diets and was lower than in regular hybrids only six times (out of 42). Every time this trait was recorded, increase of body weight was observed in cattle fed *bm3* silage, with an average daily gain reaching 158 g per cow per day. In another data synthesis, Ferraretto and Shaver (2015) pointed out a 1.1 kg per cow per day greater intake of dry matter and a 1.5 kg per cow per day greater milk yield for cows fed *bm3* diets. In addition, differences in diet intakes appeared higher in experiments comparing isogenic hybrids than in experiments with not isogenic hybrids. However, these latter experiments most often corresponded to more recent investigations, with different maize germplasm but also more performing cows having with the regular diet an intake 3.3 kg per cow per day higher than in isogenic and older experiments. Differences in milk production between regular and *bm3* diets were nearly similar in isogenic and not isogenic investigations, despite the fact that milk yields were 8.9 kg per cow per day higher in more recent and not isogenic maize hybrid experiments than in older ones.

The primary benefit of the *bm3* mutation in dairy cattle feeding is an increased silage intake. The greater efficiency of *bm3* hybrids is thus all the more valorized as maize silage is a more significant ingredient in the diet. Moreover, a similar addition of concentrate supplements to regular and *bm3* maize silages reduced the advantage of *bm3* maize over regular maize to a significant level, reducing the *bm3* silage extra intake. Most comparisons done since 2005 were based on *bm3* diets comprising less than 45% maize silage, thus leading very likely to underestimate of *bm3* silage interest. In addition, substitution of concentrates by *bm3* corn silage will be all the more possible as energy values of best *bm3* experimental hybrids have been shown nearly equal to 1.00 UFL (7.11 MJ), a value similar to the one of small grain cereals and only a little lower than that of corn grain (1.10 UFL or 7.72 MJ).

The regulation of cow appetite when they are fed corn silages is considered, above all, a result of physical regulations, as palatability traits are probably of very limited importance in properly prepared silages. Intake limitation results from the positive relationship between feed bulk density and ruminal physical fill. When the rumen has reached its maximum physical fill, digestion and movement of digesta out of the rumen must occur before cow could get new intake capacities. Intake of forage is thus mainly controlled by the time the forage is retained in the rumen (Minson and Wilson, 1994; Jung and Allen, 1995). Chewing during eating and ruminating is responsible for most of this particle breakdown in chopped forages (Minson and Wilson, 1994). Variations in time spent chewing and ruminating due to variation in tissue

mechanical resistance, and variations in tissue degradability and degradation rate by rumen micro-organisms, both explain silage intake variation in dairy cattle probably at a similar extent. Higher tissue friability and higher cell wall degradability thus underlie the higher energy value of *bm3* hybrids.

Comparisons involving the other different maize brown-midrib genes with meat or dairy cattle are very rare. In one experiment with fattening bulls, a *bm1* hybrid was slightly more efficient than its normal counterpart, but much lower efficient than its *bm3* counterpart (Barrière et al, 1994). On the contrary, *bmr6* sorghum, which are also CAD2 mutants, have a significant development in southern Europe but the average energy value of ensiled sorghum is significantly lower than the one of maize silage. The interest in cattle feeding of *bm2* and *bm4* corn hybrids has seemingly not been investigated, but these two genes have seemingly too great depressive effects on plant vigor together with a limited improvement of cell wall digestibility. The use of the two recently discovered *bm5* and *bm6* alleles should be considered as a promising strategy in silage maize breeding. Preliminary results have indeed shown that these two mutants and especially *bm5* induced important increases in *in vitro* cell wall digestibility, possibly higher than what is observed with *bm3* plants.

Perspectives in *bm3* and brown-midrib hybrid breeding and use in dairy cattle feeding

Cancellation of milk quotas and milk market liberalization in the EU strengthen the necessity of reducing cost production, including feeding costs. The availability of *bm3* hybrids on the seed market in the USA, especially developed by the Mycogen and Pioneer companies, has proved the possible breeding and use of the *bm3* mutation for cell wall digestibility and feeding value improvement in commercial hybrids, at least for late or medium late hybrids. In EU, most maize hybrids used for silage are early and medium early hybrids. The lack of development of *bm3* hybrids in European countries could relate to the higher cell wall digestibility of early germplasm in comparison to late genetic backgrounds. However, current early dent lines are most often related to the lodent group and early flint are often introgressed of BSSS or other late origins, with in addition the increasing importance of early dent hybrids. These genetic changes, occurring at the expense of older Minnesota and Wisconsin dent and European flint germplasms, contributed to the lower energy value of modern early hybrids in comparison to those registered before 1975 and could thus strengthen the interest of breeding early *bm3* (and brown-midrib) silage maize hybrids.

However, brown-midrib maize hybrids are still relegated to theoretical purposes in the EU, as their improved nutritional qualities are currently not considered to overcome the (supposed) associ-

ated deficiencies including reduced yield, increased lodging, and increased diseases, pests, and stress susceptibilities. Nevertheless, targeted plant breeding of *bm3* elite lines will very likely allow continued improvements in yield of *bm3* maize silage, as it is shown with the recently released *bm3* US hybrids. The greater susceptibility to lodging of *bm3* plants, which is generally assumed, is indeed not established and is likely not true, all the more as lodging susceptibility is primarily related to insufficient root growth. The first *bm3* experimented hybrids corresponded to reconversions of old normal hybrids that were susceptible to lodging, thus giving a poor reputation of brown-midrib germplasm. Conversely, a higher incidence of stalk breakage at grain maturity (after silage harvest) in *bm3* plants compared with normal ones often exists and an average 22% decrease in crushing strength has been shown in old *bm3* hybrids in comparison to their normal counterparts (Zuber et al, 1977). This latter fact is an illustration of the genetic determinant of *bm3* silage intake by cows, likely corresponding to the higher friability during chewing of *bm3* tissues. A negative consequence of the greater softness of *bm3* tissues is possibly the greater susceptibility of *bm3* maize to borers. Several colocalizations between QTLs for cell wall digestibility and for stalk tunnelling tolerance to European and Mediterranean corn borers have thus been shown in investigations with normal hybrids (Barrière and Courtial, unpublished data). However, not all brown-midrib genes or not all other cell wall mutants could be considered as inducing significant higher borer susceptibility.

Only combined selection within *bm3* germplasm will aim at reducing these negative correlations with the progressive accumulation of genetic factors enhancing pest tolerance without too much unfavourable effects on quality traits. With normal lines of good standability, giving hybrids with potential farm yields higher or equal to 14-16 t ha⁻¹, it is conceivable to breed related *bm3* lines giving hybrids whose yield will be reduced by only 1 to 3 t ha⁻¹, but whose cell wall digestibility will be increased by 6 to 12 percent points (Tables 2 and 3). The lower maize yield, associated with a higher silage intake, will necessitate a little larger cropping area in order to satisfy animal feeding needs, a disadvantage economically overcome by the significant reduction of energy concentrates used in the diets. In addition, the choice of using lower yielding hybrids of higher feeding value could contribute to more friendly environmental conditions of plant cropping and cattle rearing. The water need of plants is linked to its yield and plant yields have to be adjusted to present and future water availability and a reduced water requirement nearly equal to 25 mm ha⁻¹ is possible per each t ha⁻¹ reduced yield.

Breeding “brown-midrib-like” hybrid based on monolignol gene disruption or deregulation

Searches for lower lignin contents and modified structures, and improved cell wall degradability, both for cattle rearing or industrial purposes have been also investigated through transgenic approaches, especially in dicotyledonous forage (alfalfa) and woody species (poplar), in addition to those developed in Arabidopsis. Down- or up-regulation of many of the genes involved in monolignol and wall carbohydrate biosynthesis, as well as with genes regulating secondary wall assembly, have allowed obtaining plants with reduced lignin contents, altered lignin compositions and structures, modified cellulose organization and crystallinity, and modified hemicelluloses and pectins (Abramson et al, 2010).

In maize, in addition to the nonsense brown-midrib mutations of the ZmCOMT and ZmCAD2 genes, numerous other genes should be considered as targets of interest in the monolignol and *p*-hydroxycinnamic acid pathways and their upstream regulation. While ZmCOMT and ZmCAD2 are undoubtedly the main 5-OH-coniferaldehyde-O-methyltransferase and cinnamyl alcohol dehydrogenase involved in the monolignol pathway, lignin composition and occurrence in *bm1* and *bm3* mutant plants prove the existence of alternative OMT and CAD activities, either through paralog (CAD) or other OMT encoding genes. At least eleven enzymes are necessary to catalyse the different steps from phenylalanine to monolignols, plus at least three others which are specific of the pathway from feruloyl-CoA to feruloylated arabinoxylans. Search for all genes of the monolignol pathway, mainly based on the B73 sequence, has correlatively established that one or most often several paralogs exist for each enzymatic step, except for ZmCOMT (Courtial et al, 2013). The respective importance of each paralogs, as well as their possible spatio-temporal regulation is not fully elucidated, but several other genes than ZmCOMT and ZmCAD2 are likely relevant target in breeding for improved cell wall degradability.

Variable consequences on phenolic compounds are likely induced by mutations at each step of the lignin pathway, and not all are probably associated with a brown-midrib phenotype as for ZmCOMT and ZmCAD2. The old INRA line F4, bred at INRA Versailles in the late 1940's in the variety «Etoile de Normandie», does not exhibit a brown-midrib phenotype but this line is typified by a high cell degradability in *per se* value, equal or even higher than the ones of *bm3* lines. Comparative gene expression investigations have shown that, in the below-ear internode at silking stage, the ZmPAL gene (ZmPAL3a, Zm00001d017274) had an expression reduced in F4 to 18% of the expression value observed in the reference line F2, in which this gene was highly expressed (Guillaumie, 2006). The role of the ZmPAL gene under-expression in the high digestibility of the F4 line

needs further investigations at least because several ZmPAL paralogs are present in the maize genome. However, as a tentative conclusion, deregulation of ZmPAL gene(s) could probably be an efficient strategy in maize breeding for an improved feeding value, especially as PAL enzymes catalyze the first step of the monolignol pathway.

The search for the best target genes in silage maize breeding has also been considered through transgenic gene deregulation. First transgenic cell wall investigations dealt with the ZmCOMT gene and corroborated data obtained in *bm3* plants. Reduced content in lignins, reduced S units, together with higher cell wall digestibility was shown in ZmCOMT-antisense progenies (Piquemal et al, 2002; He et al, 2003), with an effect of the genetic background in which the ZmCOMT-antisense event was introgressed (Pichon et al, 2006). ZmCAD2 down-regulated plants, with less severely reduced enzyme activity than in *bm1* plants, did not exhibit the brown-midrib phenotypes and had very little reduction in lignins, but nevertheless had altered lignin biosynthesis and more degradable midribs and stems (Fornalé et al, 2012). ZmCCR1 down-regulated plants had reduced lignin content by nearly 8% and improved NDF *in vitro* digestibility by nearly 30%. In addition, down-regulated plants exhibited a brown (to dark reddish) coloration in leaf midribs and stems (Park et al, 2012), while no natural ZmCCR1 mutant has been described, based on investigations in collections of brown-midrib mutants. Down-regulated ZmCCoAOMT1 (Zm00001d036293) plants had a 22.4% decrease in lignin content and had lignins with a 57.1% higher S/G ratio, with reduced S content (Li et al, 2013). The latter result could corroborate a priority biosynthesis of G unit from feruloyl-CoA, after a ZmCCoAOMT1-catalyzed methylation of caffeoyl-CoA, while S units could originate from activity of another ZmCCoAOMT enzyme(s). However, it was also suggested by Guo et al (2001), based on CCoAOMT down-regulated alfalfa, that CCoAOMT enzymes could only partly contribute to S unit biosynthesis, which could thus originate from 3-O-methylation of caffeoyl aldehyde after a CCR-catalyzed reduction of caffeoyl-CoA. No alteration of lignin content was shown in plant midribs and only very little reduction in stems of ZmC3H1 (Zm00001d043174) down-regulated plants, a result that was related to the regular expression of the C₃H₂ (Zm00001d038555) gene (Fornalé et al, 2015). However, a 1.5 fold to 4.0 fold increase in lignin H monomer was observed, in agreement with the position of C3H genes in the monolignol pathway.

In addition to EMS chemical mutagenesis and TILLING strategies, the transposon-tagging approach (transposon mutagenized plants) is also in maize a promising strategy in the search for genes inducing significant improvements in cell wall digestibility. A ZmCCR1 such obtained mutant had thus an improved cell wall digestibility by 120%, with reduced

releases of H and G monomers and increased release of S monomers after thioacidolysis (Tamasloukht et al, 2011). This ZmCCR1 mutation resulted from the Mu transposon insertion in the first intron and only induced an incomplete down-regulation of the ZmCCR1 gene, possibly explaining that no brown-midrib phenotype was observed, contrarily to what was shown in the Park et al (2012) experiments. In addition, transposon-mutagenized mutants of the ZmCCoAOMT2 and ZmC4H1 genes were shown with an improved cell wall digestibility by 108 and 112%, respectively, while the cell wall digestibility of a ZmG6DH mutant was reduced to 90% of that of the isogenic normal line (INRA and Génoplante unpublished data).

Transcription factors as possible targets in breeding “brown-midrib-like” hybrids

In addition to genes of the monolignol pathway, upstream regulators and especially several MYB and NAC genes which are cell wall specific transcription factors should be considered as putative targets, all the more as members of these gene families were shown colocalizing with lignin and/or cell wall degradability QTLs (Courtial et al, 2014; Barrière et al, 2015; Barrière et al, 2016).

The ZmMYB42 (or ZmMYB073, Zm00001d053220, bin 4.09, pos 220.72 Mbp) gene could thus be considered as a promising target. This ZmMYB42 gene is orthologous to EgMYB1 which encodes a negative regulator of lignin related genes (Legay et al, 2001) and it was shown to repress the phenylpropanoid pathway in Arabidopsis transformed plants (Sonbol et al, 2009). In addition, the ZmMYB46 (or ZmMYB146, Zm00001d023931, bin 10.03, pos 29.36 Mbp) is ortholog of AtMYB46, AtMYB83, and EgMYB2 which were shown to be the master genes regulating as activator numerous genes involved in the secondary wall biosynthesis (Goicoechea et al, 2007; Zhong et al, 2007; Kim et al, 2014; Ko et al, 2014). This ZmMYB46 transcription factor closely colocalized with cell wall digestibility QTL having a Lod value equal to 13.7 (Barrière et al, 2008; Barrière et al, 2016).

Among NAC transcription factors, which act as master regulators of expression of several transcription factors and/or genes involved in secondary cell wall biosynthesis (Zhong et al, 2011), the ZmSWN2 (Zm00001d049678, bins 4.04, pos 39.73 Mbp), ZmSWN4 (Zm00001d002934, bin 2.03, pos 27.12 Mbp), ZmSWN5 (bin 1.01, Zm00001d027459, pos 5.71 Mbp), and ZmSWN6 (Zm00001d002828, bin 2.03, pos 24.00 Mbp) genes could be preferential targets for deregulation and searches for mutants, based on their role in different species and in maize their colocalizations with cell wall digestibility QTLs in several RIL progenies (Barrière et al, 2015).

Ferulate related genes as possible target in breeding “brown-midrib-like” hybrids

Cross-linkages through ferulate and diferulate bridges greatly impede cell wall carbohydrate degradation and their role have been «tentatively estimated to account for nearly one half of the inhibitory effects of lignin on cell wall fermentation» (Grabber et al, 2009). Ferulic units are primarily esterified to glucurono-arabinoxylans, and lignins and arabinoxylans are secondarily bridged through ferulate ether-linkages at the β -position of G units. Ferulate linked to arabinosyl side-chains of arabinoxylans also provides the way for bridging between polysaccharide chains. Over 50% of wall ferulates can undergo dehydrodimerization/trimerization and arabinoxylans are thus extensively cross-linked by ferulate di/trimerization in mature secondary walls (Grabber et al, 2004). In addition, ferulates were also shown to be esterified to coniferyl and sinapyl alcohols and thus incorporated in the lignin polymer (Karlen et al, 2016), but the possible effect of this incorporation, as well as possible genetic variation in the intensity of incorporation, are still unknown.

Illustrating the efficiency of breeding for ferulate related traits, the *sfe* mutant, which was identified after screening a set of nearly 12,000 transposon-mutagenized plants, had a 50% reduction of ferulate ester concentration in seedling leaf tissue (Jung and Phillips, 2010). The *sfe* mutant was backcrossed in the W23 line. The silages of the two progenies W23sfeM04-4 and W23sfeM04-21 were then fed to dairy cows (Jung et al, 2011). Ferulate esters and ethers were reduced by 15 and 25% in W23sfe silages compared to W23 silages, respectively. No clear changes were shown in lignin content with a tendency to a little higher content in W23sfeM04-4 in comparison with W23 and W23sfeM04-21. Ferulate ethers were reduced to 76 and 73% of the W23 value in W23sfeM04-4 and W23sfeM04-21, respectively. No changes were shown in cell wall xylose and arabinose contents. Similarly, cell wall digestibility was the same in W23sfeM04-21 and in W23, but the one W23sfeM04-4 appeared a little lower (Jung et al, 2011). Diet intake, containing nearly 39% maize silage, were greater by 1.6 kg per cow per day for cows fed W23sfe silages than for those fed W23 silage (21.8 kg per cow per day), while milk production (3.5% FCM) was 1.2 and 2.3 kg per cow per day greater for cows fed diets with W23sfeM04-4 and W23sfeM04-21 silages, respectively, than for cows fed diets with W23 silage (41.8 kg per cow per day). These results would strengthen a more significant role of the current alteration of ferulate cell wall cross-linkages on intake by dairy cattle (and correlatively on milk yield) than on plant digestibility, possibly through induced modifications of tissue stiffness and friability. The genetic determinant underlying the *sfe* mutation is still unknown. However, the obtained results in dairy cow feeding with *sfe* silages highlighted that

intake variation in cattle might be driven by a simple maize determinant which is not brown-midrib and seemingly not monolignol-related (even if the monogenic character of the *sfe* mutation is not definitely established).

In search for silage maize having higher feeding value in dairy cows, results obtained with the low ferulate *sfe* mutant also confirm the priority interest of understanding the pathway between feruloyl-CoA and feruloylated arabinoxylans, together with the upstream regulation of arabinoxylan feruloylation and ferulate cross-linkages. All available results strengthened a feruloyl-CoA origin of linked ferulate and that the ferulate transfer to arabinoxylans or UDP-arabinose is catalyzed by an enzyme encoded by a member of the BAHD family (clade A). In rice, down-regulation of such BAHD genes was associated with a 19% reduced ferulate release from leaves of deregulated plants relative to the control (Piston et al, 2010). In *Brachypodium*, RNAi lines under- and over-expressing the BAHD *Bradi2g43520* (*BdAT1*) gene showed decreased (up to 35%) and increased (up to 47%) releases of ferulate monomers and dimers from stem tissues, respectively (Buanafina et al, 2016). Considering expression in maize stems and in the ferulate-rich pericarp tissue, five members of the BAHD family could be considered in maize, including in the subgroup I the two *BdAT1* orthologs [*Zm00001d044475* (bin 3.09, pos 229.13 Mbp) and *Zm00001d011349* (bin 8.05, pos 147.54 Mbp)], two BAHD of the subgroup III [*Zm00001d037619* (bin 6.05, pos 132.05 Mbp) and *Zm00001d009960* (bin 8.03, pos 92.43 Mbp)] and one BAHD of the subgroup IV [*Zm00001d039535* (bin 3.02, pos 7.48 Mbp)], this latter one being one of the most probable candidate (Chateigner-Boutin et al, 2016). Genomic positions of these five maize *ZmBAHD* genes in the maize genome corresponded to a common genomic area located of the ancestral grass chromosome 5 (Murat et al, 2010). This fact could thus indicate that the five maize genes originated from a unique ancestral grass gene. In order to increase cell wall digestibility and/or reducing stem stiffness, any of these five considered *ZmBAHD* genes could be a relevant target for deregulation (and mutation). In addition, ferulic acid oxidative coupling appeared driven by peroxidase(s), with nine genes having a high expression in the maize ferulate-rich pericarp tissue (Chateigner-Boutin et al, 2016). However, occurrence and frequency of ferulate cross-linkages in maize secondary wall do certainly not only depend on BAHD or peroxidase gene activities, and other genes and mechanisms (Hatfield et al, 2017) should be considered. Anyway, despite the limitations of current solvolytic methods in etherified ferulate estimates (Grabber et al, 2000) and despite it could be questioned how their release and observed content is not (fully) free of lignin content and structure, the correlation between etherified ferulate release and cell wall degradability was always shown

to be negative, both in maize and in other grasses, with effect on intake and digestibility in dairy cattle.

Conclusions

The biological conversion of cell wall carbohydrates, mainly located in the secondary lignified plant cell walls, into fermentable sugars by rumen microorganisms, is hindered by their embedding with lignins, as well as by *p*-hydroxycinnamic acid cross-linkages between wall components. Improvement of maize energy value for dairy cow feeding can be based on breeding lines with high cell wall degradability values. QTL and GWA investigations have shown that cell wall digestibility is a quantitative trait involving numerous genomic locations. Moreover, respective effects of each determinant often depend on the considered genetic backgrounds, with possible variable negative effects on plant agronomic value. The use of mutants of high cell wall digestibility is thus a relevant alternative strategy in breeding silage maize for improved feeding value. A mutant gene, as well as a transgene gene, with major effect on cell wall degradability can indeed be easily and rapidly backcrossed in elite germplasm using specific markers, with simultaneous breeding against unfavorable effects.

Silage made from *bm3* hybrids, and very likely of other brown-midrib and numerous «brown-midrib-like» hybrids, allows an increased voluntary intake by dairy cattle, together with an increased and faster cell wall digestion, as compared with non-improved maize silage. High-producing dairy cows have most often a negative energy balance during the early period and peak of lactation, because their voluntary intake is not sufficient to satisfy the high energy demands corresponding to their milk production. The higher intake and digestibility of brown-midrib genotypes allow reducing the energy disequilibrium during the beginning of lactation and also allow formulating diets with higher forage to concentrate ratio. These characteristics of brown-midrib hybrids let to reduce the giving of costly energy concentrates, and to lower the starch content in the diet, thus contributing to reduce the risks of subacute ruminal acidosis (with consequently reduced veterinary costs). The higher feeding value of brown-midrib hybrids could be partially offset by the still lower yield associated with this trait. However, current communications given by US companies selling *bm3* hybrids show a similar yield in these hybrids as in normal hybrids of similar earliness. Moreover, forage yield has now to be strongly related to water availability, with reduced interest of still greater yields, but focusing now on hybrid tolerance to hot and dry periods, in addition to greater feeding value. Despite the little supply of land possibly requested for forage production, the brown-midrib(-like) traits have been shown to be beneficial for farmers, due to concentrates and extra costs saving, and higher milk production. In addition, it has been shown that feeding cows with brown-midrib silages does not

increase methane emissions despite their higher fiber digestibility as compared to regular silages. Moreover, methane production was lower for cows fed the *bm3* diets when it was based on gross energy intakes or on milk yields. (Schwam et al, 2015; Hassanat et al, 2017). If taking simultaneously into account the reduced need of energy concentrates, feeding cows with brown-midrib hybrids can thus lead to reduced environmental costs per animal or milk unit.

Despite the large development of herbicide and insect tolerant transgenic maize hybrids on large seed markets, maize transgenic genotypes with improved cell wall digestibility and energy value have been only investigated in experimental conditions, without commercial development. The lack of release of cell wall genetically modified hybrids on the different seed markets could be related to the facts i) that transgenic plants are currently restricted in several world areas, including EU, with costly regulatory hurdles, ii) that, in addition, registration rules and/or marketing objectives of maize for silage use do not take sufficiently into account the hybrid feeding value, iii) that cell wall degradability of maize silages has an average higher value than that of dicotyledonous forages developing secondary cambium, and iv) that the current pedigree ways of breeding had allowed preserving maize hybrid energy value, even if no real improvement was attained in comparison to hybrids of the 1980' and that feeding value of several older European varieties was higher than the one of the currently cropped hybrids.

The development of the CRISPR/Cas9 editing technology allowing targeted modifications, together with the use of tissue-specific promoters and paralog-specific down-regulations, will allow impairing the monolignol or ferulate biosynthesis, and likely carbohydrate changes, in specific tissues or cell-types. Such strategies should allow the release of plants having similar improved cell wall degradability and/or intake in cow than with brown-midrib lines, but without at least a part of negative side-effects possibly linked to these mutations. It was thus suggested that plants can tolerate large decreases in lignin content when decreases are targeted to specific cell types (Chabannes et al, 2001; Jung et al, 2012), especially when preserving vessel lignification in order to avoid collapse risks during hot and dry periods. Moreover, an improved edited or transgenic (including brown-midrib) hybrid would not always require that both parental lines be homozygous for the modified gene as it is the case in (natural) recessive mutations.

Frequent critical opinions of both corn breeders and farmers regarding brown-midrib plants will probably soon significantly change. The greater understanding of cell assembly and lignification together with the identification of a set of key-genes and their spatio-temporal regulations will allow moving from a limited number of brown-midrib mutants to a larger panel of genetic resources for silage maize improve-

ment, with reduced negative side-effects. These progresses should provide the release of new hybrids fitting well with energy needs of dairy cows in environmentally sustainable conditions of rearing.

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