

INVITED REVIEW ARTICLE

Trends in new technological approaches for forage crop improvement

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Abstract

Forage crop production constitutes an important arena of agricultural research and caters to the food and feed need of the booming global dairy and livestock industry. Conventional and traditional plant breeding have been the most commonly used approach for the forage crop improvement across the planet. However, with the recent surge of the technological innovations in life sciences research, new approaches are now being regularly employed for the forage crop improvement in conjunction with the evergreen plant breeding techniques. Not just forage industry, the recent trends also indicates towards the increasing popularity of certain species for biofuel production too. This review humbly captures some of the latest molecular, biotechnological, tissue culture and genetic engineering approaches that are being tried in improving forage crop production in recent times.

Keywords: forages, breeding, selection, plant biotechnology, genetic engineering, molecular, tissue culture, genomics.

Abbreviations: ADH: Alcohol dehydrogenase; AMGT: *Agrobacterium*-mediated gene transfer; AMV: Alfalfa Mosaic Alfamovirus; CAD: Cinnamyl Alcohol Dehydrogenase; CPPs: Carrier Peptide Proteins; CTBI: Canadian Triticale Biorefinery Initiative; DESM: Direct Somatic Embryogenesis for monocots; EST: Expressed Sequence Tag; EU: European Union; FFG: Forage Functional Genomics; GEM: Germination of Monocots; GM: Genetically Modified; GUS: β -glucuronidase; IGPs: International Genomics Projects; IMC: Isolated Microspore Culture; miRNA: microRNA; MS_{reg} Medium: Murashige-Skoog Regeneration Medium; PIs: Protein Inhibitors; PPGP: Pasture Plant Genomics Program; RM: Rooting Media; RNAi: RNA interference; SEM: Secondary Embryogenesis of Monocots; siRNA: small interfering RNA; Tat: Tat monomer; Tat₂: Tat dimer; WCMV: White Clover Mosaic Potexvirus.

Introduction

A very recent surge in the forage crop improvement has been in a very newly emerging research area collectively known as Biofuel or Bioenergy research (Jaradat *et al.*, 2010). It is an extension of traditional forage improvement restricted to animal feed to production of energy crops (Ghorbani *et al.*, 2011). Sanderson and Adler (2008) consider the forage lignocellulose as the “second generation biomass feedstock” for transforming into “energy-related end products”. The most commonly worked on forage species in the biofuel industry are switchgrass (*Panicum virgatum* L.), reed canarygrass (*Phalaris arundinacea* L.) and alfalfa (*Medicago sativa* L.) (Al-Ghumaiz and Motawei 2011; Ghorbani *et al.*, 2011). The greatest advantage of utilizing forage crops as biofuel sources are that the farmers are already familiar with the breeding and agronomics of these crops and are often regularly incorporated in their crop rotation practices making it both beneficial for their farming practices as well as economically rewarding for potential opening up of new and emerging markets (Basu *et al.*, 2007; Zhang *et al.*, 2010 Al-Ghumaiz and Motawei 2011; Lithourgidis *et al.*, 2011).

In addition to this, another extension and diversification of the forage industry has been towards catering to the needs of the newly emerging but rapidly expanding regional and global nutraceutical and functional food industries (Basu *et al.*, 2007). Several forage crops such as alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), fenugreek (*Trigonella foenum-graecum* L.) are rich in different

phytochemicals that have important medicinal, pharmacognostic and clinical properties and are regularly needed for different formulations and products generated by nutraceutical and functional food industries (Acharya and Thomas, 2007). Such new developments have opened up promising opportunities for researchers involved in forage crop species improvement programs globally. From a modest beginning of *first generation* animal feed crops, forages have now extended their applications in the *second generation* multi-billion dollar industries of turf grasses for lawns, gardens, parks, golf courses; biofuel and bioenergy initiatives; and nutraceutical and functional food industries (Fig. 1). The same forage crops grown over centuries have now multiple end users other than the animal food and feed industry alone (Jaradat *et al.*, 2010; Ghumaiz and Motawei 2011). This has clearly initiated new research interests and breathed in life into the previously neglected forage breeding programs globally. Molecular breeding based on transgenesis to defeat limitations in forage quality may be manoeuvred to improve traits comprising dry matter digestibility, water-soluble carbohydrate content, protein content, secondary metabolites, alkaloids, etc. Armed with the need of the time and emerging technological innovations rocking biological and life sciences disciplines, forage breeding has once again been transformed into a classy research domain with huge promises of high dividends in not so distant future (Zhang *et al.*, 2010; Ghumaiz and Motawei 2011). This review

explores the new arenas of modern biology and how these technologies are impacting or could influence future forage production.

Biotechnological applications

A. Tissue culture, genetic engineering and gene delivery methods

1. Tissue culture and genetic engineering

Genetic variability is a very important and integral functional component of any breeding program essential for improving desirable characteristics in forage crop species (Islam, 2010). Due to the totipotent nature of plant cells, a number of cell, tissue and organ culture protocols have been developed over time and are currently available for exploiting in forage crop improvement programs (Sikdar and Kim, 2010). In recent times, plant cell culture is considered to be a significant source of useful genetic variations and the genetic variations generated through plant tissue culture applications is termed as 'somaclonal' variations (Chawla, 2002; Jain and Brar, 2010). Different aspects of plant tissue culture have been exploited and explored by different researchers such as embryo culture, seed culture, cell culture, meristem culture, callus culture, root and shoot cultures, organ culture (nucellus and endosperm cultures), anther and ovary cultures, Isolated Microspore Culture (IMC), bud culture, protoplast culture, doubled haploid production, micropropagation etc. (Chawla, 2002; Touraev *et al.*, 2009; Jain and Brar, 2010; Sikdar and Kim, 2010). Tissue culture which is an efficient and robust plant regeneration systems and an integral part of most plant genetic transformation strategies, offers also the combination of whole or partial genomes by somatic hybridization and cybridization through protoplast fusion (Forster *et al.*, 2000; Spangenberg *et al.*, 2000). Somaclonal variation, gametoclonal variation and protoclonal variation which usually bring changes in physiological mechanisms, agronomic characteristics, plant type, and resistance to biotic and abiotic stress conditions, have been proved to be useful methods for creating desirable genetic variation in most cereal and forage crops (Rush *et al.*, 1998). Larkin *et al.* (1984) followed by other researchers suggested somaclonal variation as a source of novel variation for plant improvement demonstrating heritable and stable somaclonal variation in wheat. Researchers have mainly pointed out two phenomena; pre-existing genetic variation among cells in the explant tissues and variation induced due to difference in cultural environment, which may result in clonal variations (Karp and Bright, 1985; Scowcroft and Larkin, 1988; Brar and Jain, 2010).

Although the causes of somaclonal variation are yet to be understood properly, chromosome rearrangements, chromosome deletions, single base-pair changes, changes in expression of multigene families, alteration in sequence copy number, alteration in ploidy level, changes in activity of transposable elements, alteration in gene amplifications, etc. are considered as known causes of somaclonal variations (Lee and Phillips, 1988; Brown, 1989; Larkin *et al.*, 1989; Sikdar and Kim, 2010). Somaclonal variation has been reported as a useful tool for disease resistance in wheat, barley, maize, oat and rice through *in vitro* selection for changes in disease resistance (as reviewed in Harms, 1992). In wheat somaclonals, Oberthur *et al.* (1993) found two independently inherited dominant leaf rust resistant genes

which were absent in the parents of the explants. Somaclonal variation was found functional to cause variation in mitochondrial DNA in somaclones of triticale and wheat (Hartmann *et al.*, 1994; Weigel *et al.*, 1995) and changes in gliadin proteins in case of *in vitro* regenerated wheat plants (Cooper *et al.*, 1986; Obukhova *et al.*, 1991; Sikdar and Kim, 2010). *In vitro* selection in wheat somaclones for abscisic acid insensitivity resulted in the development of two somaclones (KTC86211 and KTC86424) having significantly delayed senescence, greater kernel weight and higher grain yield than the parent cultivar (Lu *et al.*, 1989). Larkin *et al.* (1984), Maddock and Semple (1986) and Mohmand and Nabors (1990) reported stable somaclonal variation in wheat for beneficial agronomic traits, while Bhaskaran *et al.* (1987) reported stable somaclonal variation for agronomic traits in sorghum. In another study, Smith and Bhaskaran (1988) showed these agronomic traits in sorghum somaclonals were inheritable in advanced generations. Development of somaclonal lines of sorghum resistant/tolerant to biotic and abiotic stresses were also reported by many researchers (Waskom *et al.*, 1990; Isenhour *et al.* 1991; Miller *et al.*, 1992; Jain and Brar, 2010; Wagiran *et al.*, 2010). Apomixis, a mode of reproduction in which progeny are exactly similar to the female parent, is a limiting factor to genetic improvement through hybridization.

Many forage grasses are apomictic that limits the variability within natural populations and populations produced through conventional breeding programs. Somaclonal variation may be a practical alternative to generate new and unique improvements in apomictic species (Rush *et al.*, 1998). Ahloowalia (1983), and Jackson and Dale (1989) reported variation in morphological traits, such as leaf and spikelet size and shape, floral development and vigour in ryegrass (*Lolium* sp.) somaclones. The above mentioned researchers also observed chromosome alteration including changes in number though those were not always correlated with phenotypic changes. Bajaj *et al.* (1981), and David and Cohen (1992) evaluated variation in case of *in vitro* regenerated *Panicum* sp. and dallisgrass (*Paspalum dilatatum* Poiret), respectively. In another study, dallisgrass somaclones were field tested and selected for determination of forage and seed yield. Improvements in agronomic traits were found in the somaclonals (Croughan *et al.*, 1994). The production of somaclones with specific chromosome changes in case of *in vitro* regenerated tall fescue (*Festuca arundinacea* Schreb.) plants has been reported by several researchers (Reed and Conger, 1985; Eizenga, 1989; Dahleen and Eizenga, 1990; Jain and Brar, 2010; Basu *et al.*, 2011). Somaclones derived from two subcultures of a ryegrass (*Lolium multiflorum* Lam.) X tall fescue (*Festuca arundinacea* Schreb.) hybrid were evaluated by Kasperbauer *et al.* (1979). Plants originated from first culture were phenotypically and cytologically same as explant source, but plants derived from older culture were different in phenotype and ploidy level from the source (Basu *et al.*, 2011). Sometimes, somaclones may look and grow identical to the parent, but changes in metabolic function may have occurred which can be revealed by biochemical analysis (Mohamed *et al.*, 1992). Winicov (1991) found salt tolerant cell lines of alfalfa (*Medicago sativa* L) selecting on tissue culture medium containing 1% sodium chloride with improved salt tolerance compared to parent (Basu *et al.*, 2011). Another approach that we will discuss is used to induce direct somatic embryogenesis, secondary embryogenesis and regeneration of seven species of fertile green cereal plants including several potential cereal

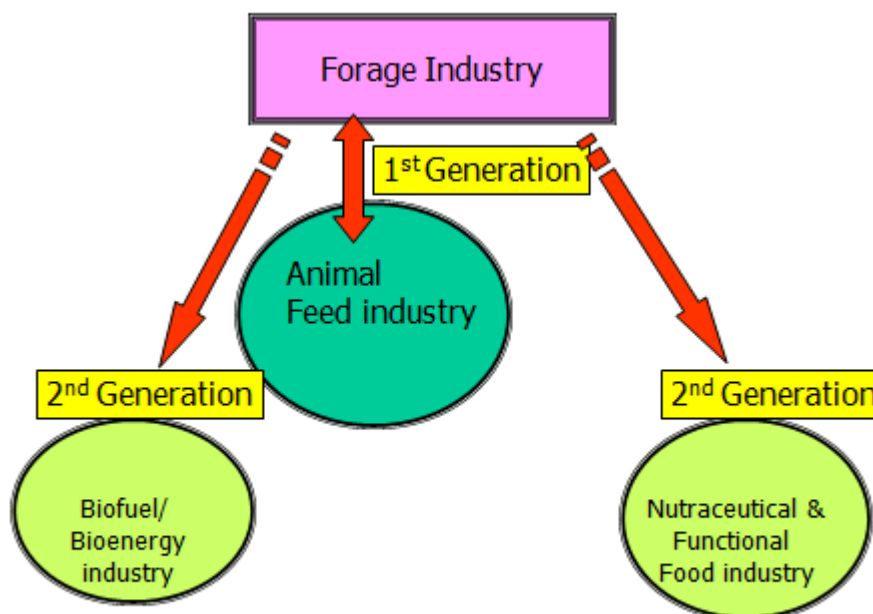


Fig 1. The transition and evolution of forage industry from first to second generation.

members in Western Canada (Eudes *et al.*, 2003). In brief, the protocol involves rapid induction of direct somatic embryogenesis of monocots (DESM) from excised immature scutellum on a specific medium abbreviated as DSEM medium. In the next step, newly emergent globular embryos from the excised scutellum were subjected to another round of secondary embryogenesis of monocots (SEM) by growing them on a second specific growth media for secondary embryogenesis media abbreviated SEM, followed by germination of monocots (GEM) in a GEM media and subsequent regeneration in Murashige-Skoog medium (MS_{reg} medium). Finally young regenerated embryos are subjected to growth for root development in the Rooting Media (RM) followed by their growth in root trainers and pots in the green house. Most commonly gene delivery approaches with respect to biotechnological improvement of forage crop species such as alfalfa has been either using *Agrobacterium*-mediated gene transfer (AMGT) protocols or via biolistics or Helios[®] gene gun delivery method (as reviewed in Bhowmik and Basu, 2008). An additional media called DSEM mannitol is used in between DSEM and SEM when genetic transformation is targeted via biolistics or gene gun mode of delivery of important transgenes. Eudes and Amundsen (2005) reported IMC protocols for hexaploid triticale that are now being currently used to exploit significant success rates in several cereal members. This research group identified significant interactions for the number of embryos and calluses produced, green and albino plantlets generated and fertility levels of generated green plants. The researchers reported 22% of total lines generated to be fertile and doubled haploids. This is a new approach for triticale breeding involving the dual technology of *in vitro* selection and genetic engineering. The success of this group has important implications of forage cereal crops improvement too. The most important reason for the potential successes being the fact that such genetically engineered and tissue culture derived doubled haploid plants take less time to be developed compared to conventional breeding approaches and also helps in inter-kingdom transfer of important gene(s)

for improving or introducing desirable agronomic characteristics in target forage crop species. Such agronomic characters may be genes for pest or disease resistance, herbicide or fungicide resistance, draught, cold or salinity resistance genes or growth parameters such as early maturation, early or late flowering, rapid nodulation in case of forage legumes, improvement in yield attributes like biomass and seed yield improvements etc.

2. Gene delivery approaches

In addition to conventional gene delivery approaches such as biolistics, AMGT, protoplast fusions, a fairly recent gene delivery approaches tried by using Carrier Peptide Proteins (CPPs) in delivering reporter genes in wheat and triticale (Chugh and Eudes, 2008; Chugh *et al.*, 2009). This research group for the first time demonstrated high uptake levels of different synthetic CPPs in the permeabilized immature embryos of wheat (Chugh and Eudes, 2008). Among CPPs used by this group, Tat monomer (Tat) exhibited highest fluorescence uptake (4.2X) in permeabilized embryos compared to the negative control used in the experiment (mutated-Tat). Their glucuronidase histochemical assay reveals that CPPs can effectively deliver transcriptionally active β -glucuronidase (GUS) enzyme in permeabilized immature embryos. The researchers also reported that low temperature; endocytosis and macropinocytosis inhibitors reduce the Tat₂-GUS complex. Permeabilized embryos transfected with Tat₂-plasmid DNA complex showed 3.3X higher transient GUS expression; while, incorporation of cationic transfecting agent Lipofectamine[™] 2000 to the above mentioned complex resulted in 1.5X transient GUS expression. In the second study on triticale mid-late uninucleate microspore, Chugh *et al.* (2009) reported that both Tat-monomer (Tat) and dimer (Tat₂) were capable of efficiently transducing the GUS reporter gene in transcriptionally active form in 5 and 14% of the microspores, respectively. In case of another CPP (Pep-1) 31% of microspores were effective in transducing active

GUS reporter gene. The authors reported a preferred micropinocytic mode of entry of the Tat₂-GUS complex and recommended a ratio of 4:1 Tat₂- linear plasmid (pActGUS) in complex preparation for microspore transfection. About 2% triticale microspores received the Tat₂-GUS complex compared to the control. The authors suggested potential applications of CPPs in designing simple time and cost efficient plant genetic engineering protocols based on the success of their work. The researchers clearly demonstrated the efficiency of CPPs in delivering cargo macromolecules to monocot microspores. Such new gene delivery approaches may soon be exploited in a number of forage crop species too and seems to be promising for their targeted improvement.

B. Gene expression studies, plant omics and modern aspects of sequencing in relation to forage improvement

1. Gene expression

Gene expression studies have become quite commonly available on different forage species in recent times. Molecular and biochemical responses such as expression of alcohol dehydrogenase (ADH) gene to oxygen deprivation at low temperature have been now reported in forage crop species namely alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), timothy (*Phleum pratense* L.) and orchardgrass (*Dactylis glomerata* L.) by Bertrand *et al.* (2001). The authors reported lowest expression in timothy compared to the other species possibly due to enhanced resistance of timothy to anaerobic conditions at reduced temperature level associated with slow glycolytic mode of metabolism. Dry matter digestibility of forage plants has a negative correlation with plants flowering and senescence (Stone, 1994). Increasing dry matter digestibility has been ranked as the most important goal in genetic improvement of nutritive value of forage crops (Smith *et al.*, 1997). Lignification of plant cell walls, considered as the prime cause in lowering forage digestibility, increases as they mature (Buxton and Russell, 1988; Badea and Basu, 2009). Down-Regulating monolignol biosynthetic enzymes through antisense and sense suppression in transgenic forage plants are currently being explored for improved digestibility (Spangenberg, 2000; Keviani *et al.*, 2010). Transgenic antisense *CAD* (Cinnamyl Alcohol Dehydrogenase) tobacco and alfalfa plants with down-regulated *CAD* activity have been reported indicating that improvement of dry matter digestibility by the introduction of chimeric sense and antisense lignin biosynthetic genes can be achieved apparently without impairing normal development of the plant (Bernard Vailhé *et al.*, 1998; Baucher *et al.*, 1996, 1998; Badea and Basu, 2009; Brar and Jain, 2010). Genetic manipulation of monolignol biosynthesis using transgenic approaches to enhance herbage quality are currently being explored in forage legumes (e.g. *Stylosanthes humilis* Kunth, *Medicago sativa* L.) and forage grasses (e.g. *Lolium perenne* L. and *Festuca arundinacea* Schreb.) (Guo *et al.*, 2000; Spangenberg *et al.*, 2000; Brar and Jain, 2010; Li *et al.*, 2011). The increased level of soluble carbohydrates especially fructans (polyfructose molecules) appears to counterbalance the decline in digestibility due to lignification (Beever, 1993). A group of researchers have reported successful transgenic approaches for the genetic manipulation of fructan biosynthesis to enhance herbage quality and tolerance to abiotic stresses in both forage legumes, such as *Trifolium repens* L., *M. sativa* L. and forage grasses, such as

L. perenne, *F. arundinacea* (Jenkins *et al.*, 2000; Johnson *et al.*, 2000; LePage *et al.*, 2000; Lidgett *et al.*, 2000; Ye *et al.*, 2000; Goyal *et al.*, 2009; Brar and Jain, 2010; Basu *et al.*, 2011; Li *et al.*, 2011).

LePage *et al.* (2000) have reported the production of transgenic white clover (*T. repens*) expressing *Bacillus subtilis* SacB genes for enhanced drought tolerance. In another study, transgenic lucerne (*M. sativa* L.) and white clover plants expressing a fructosyltransferase gene, derived from *Streptococcus salivarius*, have also been reported (Jenkins *et al.*, 2000). Molecular response in expressing genes encoding different 'rumen by-pass' proteins rich in S-amino acids has been reported in many transgenic forage legumes (Schroeder *et al.*, 1991; Ealing *et al.*, 1994; Khan *et al.*, 1996; Brar and Jain, 2010). The authors observed low levels of expression for the ovalbumin gene in transgenic lucerne (*M. sativa* L.) and the pea albumin gene in transgenic white clover (*T. repens* L.). Recombinant DNA approaches aiming to enhance fungal disease resistance have been undertaken mainly in forage legumes, such as lucerne and clover. Coat protein mediated resistance to Alfalfa Mosaic Virus (AMV), and to both alfalfa AMV and White Clover Mosaic Potyvirus (WCMV) have been reported in transgenic lucerne (*M. sativa* L.) and transgenic red clover (*T. pratense* L.), respectively (Hill *et al.*, 1991; Kalla *et al.*, 2000). Recombinant DNA approaches have been adopted for protection of forage crops against target pests which can be controlled by Bt toxins and PIs (protein inhibitors). Expression of Bt toxins and PIs were shown to be effective against selected insect pests in transgenic forage legumes (e.g. *T. repens* L.) (Voisey *et al.*, 1994, 2001). Ikeda *et al.* (2006) have developed a nice protocol for the discrimination of forage crop seeds using ribosomal intergenic spacer analysis. This technique will certainly help plant breeders and farmers in identifying the true-bred lines from contaminating seed lines with very high percentage of accuracy.

2. Genomics

Forage breeding has now ventured into the modern 'Genomics era', more frequently being referred as the 'Omics era'. It is being very correctly said that molecular biology has introduced a wide array of tools to facilitate this rapid transition (Spangenberg, 2000; Lum and Min, 2011). In addition to the tremendous progress made in molecular biology, bioinformatics too have advanced to a cutting-edge technology stage and together these two have culminated the development of the modern Omics era in the most comprehensive ways imaginable. Recently genomics of several new or less known forage crop species are also surfacing rapidly indicating the omics revolution currently stimulating every frontier research aspects under crop sciences. A nice example is the recent publication of the genomic composition of a synthetic hexaploid cereal forage crop *Tricepiro* (2n = 6x = 42), a product of cross between hexaploid triticale (2n = 6x = 42) and octaploid Trigopiro (2n = 8x = 56) from Argentina (Ferrari *et al.*, 2005). Another example comes from Brazil in a classic review on the cytogenetical and evolutionary aspect of regional forage legumes by Schifino-Wittman (2000).

Traditionally molecular and genomic data for several forage species and turf grasses were not easily available in comparison to conventional food crops such as rice, wheat or maize (Humphreys, 2005; Lum and Min, 2011). However, recently reliable genomic and molecular data are available for

several forage and turf grass species in public domain like the ryegrass (*Lolium* spp.) and fescue (*Festuca* spp.). Significant progresses have been made in several forage and turf grass species in the context of both biotic and abiotic stress tolerances; however, the challenges facing researchers include how to pipeline these technological innovation and molecular kits and tools in a more simplified, rapid and cost-effective fashion to end users (Zhang *et al.*, 2006; Brar and Jain, 2010). Similar plight has been advocated by Varshney *et al.* (2009) regarding molecular and genomic data scarcity of several minor (rightly named as ‘orphan’ by the authors) legume crops including potential locally adapted forage species growing in the arid and semi-arid regions of the continents of Africa and Asia. Plant genomics projects with a major focus on EST (Expressed Sequence Tag) discovery are currently being undertaken for two model forage legumes, *Lotus japonicus* L. and *Medicago truncatula* Gaertn. (Cook, 1999; Cook and Denarie, 2000). Approximately 80,000 ESTs from *M. truncatula* have been generated by International Genomics Projects (IGPs) with an estimation of 100,000 sequences to be available by December 2000 (Spangenberg, 2000). In another report, Spangenberg *et al.*, (2000) reported approximately 100,000 ESTs has been generated from the key forage crops, perennial ryegrass (*L. perenne* L.) and white clover (*T. repens* L.) by a joint Pasture Plant Genomics Program (PPGP) using high-throughput sequencing of randomly selected clones from cDNA libraries representing a range of plant organs, developmental stages, and experimental treatments. Comparative sequence and microarray data analyses from ryegrass and clover with data from complete genome sequencing projects such as in *Arabidopsis* and rice would provide insight into conserved and divergent aspects of forage grass and legume genome organization and function (Spangenberg, 2000; Jain and Brar, 2010). The researches conducted on the genetics of such less known yet important species are a welcome sign for future improvement of forage crops since this will include the genomic level dissection of more and more genetically complex species and possible identifications of complex gene(s) or gene cluster(s) that could be taken advantage of introducing desirable characters. This could happen only through more and more engaging laboratories on related crops coming together on the same platform and share and exchange molecular information. The ‘genomic explosion’ has opened up an enigmatic ‘Pandora’s box’ that would spin up more genetic information than was ever expected in recent past. Strong collaborations among national, international, regional, zonal and local labs could rapidly help in filling up the lacunae of information that still haunts and plagues forage crop improvement programs globally. This is still an area under rapid development currently and would certainly see more stupendous discoveries in the coming decades (Humphreys, 2005; Touraev *et al.*, 2008; Varshney *et al.*, 2009; Zhang *et al.*, 2006; Angaji *et al.*, 2010; Brar and Jain, 2010; Islam, 2010; Keivani *et al.*, 2010).

3. Next-generation sequencing

Gene sequencing has similarly covered big strides from the days of the conventional Maxam-Gilbert and later more frequently used Sutherland approaches. Modern day genomics have benefited substantially from the recent progresses made in GENERATION NEXT sequencing technologies commonly available. According to Metzker (2009) majority of the ‘next-generation’ sequencing

technologies are not individual molecule methods any more since they are now dependent upon DNA amplification such as the Roche/454, Illumina/Solexa and Life Technologies/Agencourt Personal Genomics. Although Helicos Biosciences developed a single molecule method; however, its efficiency was limited due to its dependence on reversible terminators restricting its ranges up to analyses of short fragments. The new generation of genome sequencing technologies has been labeled as ‘third generation sequencing’ by Nature News (2009). The rapid development in sequencing technology has great implications with respect to the required time for sequencing, the expenses involved and the accuracy of the results obtained (reviewed in Pettersson *et al.*, 2009). This recent surge in genomic sequencing was initiated by the pyrosequencing technology released as Roche/454 GS20 in the year 2006 and being replaced by GS-FLX by the very next year with a five fold improvement in the data output (Batley and Edwards, 2009). Since the introduction of the second generation genome sequencing by Roche/454, Illumina has launched their GAI technology, strongly competed by Applied Biosystem’s release of SoliD sequencing technology (as reviewed in Batley and Edwards, 2009). Ever since then sequencing technologies have passed through revolutionary stages of modern improvisation and technological enrichment at an unprecedented pace. Several second generation technology platforms available as recently as in 2007 shows further sophistication, improvement and enrichment as early as 2008 (as reviewed in Gupta, 2008) clearly illustrating the fact how rapidly the technology progress is happening around us. The large scale sequencing success, lower costs and less time are all making possible the rapid publication of genome sequencing data for so many different species within a short frame of time. This is leading to unprecedented opportunities for high throughput functional genomics research and is applied to a wide diversity of research areas such as whole-genome sequencing, targeted resequencing, detection of transcription binding sites and non-coding RNA expression profiling (as reviewed by Morozova and Marra, 2008). The greatest implication of this technology surge with respect to forage genomics is that it is helping the publication of several forage cereal and legume crops across the globe. Due to the easy availability of the cutting-edge technological innovation in third generation genome sequencing the BIG void due to the lack of necessary genomic data for several important forage crop species is going to be filled up quite rapidly by the next decade. This is welcoming good news for forage breeders, forage molecular and genomics researchers and those associated directly and indirectly as primary and secondary stakeholders respectively in the rapidly expanding global forage industry! It would help forage breeders, forage biotechnologists and forage genetic engineers to pin point essential gene(s) or gene cluster(s) necessary for specific character improvements. Gene expression studies, integration pattern studies for desirable transgenes into genomes of target forage species and locating transcriptionally active sites/regions in the genomes of different forage species would be easier to locate and identify. This modern sequencing technology will be extremely convenient in establishing the much-neglected aspect of forage functional genomics (FFG). Not just that it would help in substantially improving the other significantly important research domains such as metabolomics, transcriptomics and proteomics of neglected species of forage crops.



Fig 2. Available traditional and modern forage improvement technologies.

C. Epigenetics and RNAi technology

The term ‘epigenetics’ refers to a change in gene expression induced by any mechanism other than that which induces changes in underlying DNA sequences (Tost, 2008; Angaji *et al.*, 2010). The most commonly reported mechanisms involving epigenetics are either via post-translational modifications of amino acids constituting histone proteins referred to as histone modifications, or thorough incorporation of methyl groups (-CH₃) to the DNA at CpG sites converting normal cytosine to 5-methylcytosine, a phenomenon referred to as methylation or through chromatin remodeling proteins (Boyko and Kovalchuk, 2007; Angaji *et al.*, 2010). One of the latest aspects of modern biological sciences research and is now believed to be covering all major hot areas of biological research, whether it is stem cell research or research fields dealing with cancer biology, cellular and molecular biology, molecular genetics and pathology, biotechnology, microbial, animal, plant or crop genetics, both classical and applied molecular biology and biochemistry (Angaji *et al.*, 2010). Recent epigenetics researches from those involving DNA methylation, DNA methyltransferases and methyl-CpG binding proteins to genome-wide and genome-specific methylation pattern study approaches to advances like studies dealing with histone modifications and variants, polycomb silencing, role of non-coding RNAs, genome imprinting, dynamic epigenetics and X chromosome inactivation have brought in widespread changes to traditional concepts to the realms of stem cell research, cancer biology and formidably to plant and animal traditional and conventional genetics (as reviewed in Tost, 2008). New concepts like ‘epialleles’, ‘epimutations’, ‘epigenomes’ and ‘epigenomics’ are revolutionizing and

reshaping the traditional genetical concepts in many frontiers of modern biology and domains in life sciences (Angaji *et al.*, 2010). Ability of model plants like *Arabidopsis* are being investigated in vivid details in providing comprehensive proof regarding their abilities to withstand both biotic (pathological diseases) and abiotic (draught and cold tolerances) stresses and in reshaping genome instability under stressfull and conditions critical to plant life highlighting a genetic-molecular pathway in hitherto unknown aspect of plant evolution (Boyko and Kovalchuk 2007). A detailed coverage of all these new concepts and research summary with respect to pants is beyond the scope of this review. However an important take home message is that although no recent information are available in particular context of forage crops at this point of time, since majority of the studies are currently restricted to model plants in most laboratories; but the information and technology generated will undoubtedly have influential impacts on the genetics of most cultivable crops and forage crops will be an integral part of that too. A very recent and powerful technology innovation in molecular biology and molecular genetics has been the case of RNA interference technology or RNAi technology. This is an unique system that follows a cascade reaction regulated by specific enzymes and carrier molecules and regulate different levels of cellular gene expression. There are two distinct types of small RNA molecules that are core biomolecules regulating the basic aspects of RNAi technology, namely miRNA or microRNA and siRNA or small interfering RNA.

The RNAs are direct gene products and these specific small RNAs are capable of binding to specific target RNA molecules and either upregulate (promote) or downregulate

(block) their molecular activities (Fire *et al.*, 1998). The most common impact of RNAi is blocking or modifying the targeted protein production by the corresponding messenger RNA. The RNAi system play an important role in the defence mechanism of the host cells but are most prominent in regulating different developmental pathways and overall cellular gene expression (Macrae *et al.*, 2006). Although first discovered in plants is mostly worked out in animal, mammalian and yeast systems, but has great potential role in silencing specific genes in different metabolic pathways of plants generating some desirable effects. Major research findings in forage crops are still limited; however, is expected to play major roles in gene expression of plants in not so distant future. A schematic diagram thematically representing different available traditional and modern forage improvement technologies is represented by Fig. 2.

Genetically modified forage crops and public response

There is no doubt that there is still that element of uncertainty and uncomfortable feeling among general public regarding Genetically Modified (GM) crops and GM forage crops are no exception in that list. Since final products will be processed from animals in the meat and allied food industries from animals raised on GM forage crops, the questions of foreign DNA or undesirable transgenes getting into the animal and human feed and food chains respectively is an important health concern (Small, 2004). In addition to the health aspect, media propaganda and religious and personal faiths and preferences are also an integral part of the apathy against GM crops. A fairly recent survey by Small (2004) reports detecting significant socio-cultural, spiritual and ethical oppositions to GM forage crops and negative response to consume animals fed on GM crops in New Zealand. Such reports clearly indicate that still a big part of the public opinion is against the rapid commercialization and acceptance of GM forage crops in the present day society. The response varies in intensity from geographical and cultural regions to another; however, there is undoubtedly a significant pattern of outright to moderate rejection of genetically engineered food and feed crops. A big part of the responsibility to make it more understandable and acceptable to the public before any formal decisions and/or oppositions are made lies on all of us. We need to understand the public plight and explain it more candidly in the light of most recent scientific evidences (Basu and Kovalchuk 2007; Brar and Jain, 2010). O'Brien and Mullins (2009) in their review have nicely pinpointed this critical aspect concerning GM crops development in the context of Ireland. The authors have rightly pointed out that the successful possibilities of meeting future challenges of increasing food and feed crops and enhanced agricultural productivity without impacting human and animal health and environmental quality will be largely dependent on technological innovations in the realm of genetically engineered crops.

Although there is an enormous opposition to underestimate this fact; however, no real in depth studies and survey has been conducted about that in European Union (EU), one of the strongest opponent of GM crops, assessing the potential of GM crops in the context of burning issues of recent times such as climate change, complex environmental legislations, biodiversity loss and sustainable biofuel generation. Several of the genetically engineered crops with divergent agronomic qualities and properties could serve as an essential component to the tillage sector in the context of EU

restrictions on nitrogen usage. GM food and forage crops with better protein constitution could change the trend of import duties on animal feed and crops with engineered oil or lignocellulose composition could assist in developing biodiesel and bioenergy generation at micro scale in the regional perspective (O'Brien and Mullins, 2009; Brar and Jain, 2010). Dixon (2004) suggested that the recent progress in genetically manipulating lignin, condensed tannins and saponin biosynthetic pathways will help in producing generating genetically engineered alfalfa and related forage crop species for reducing bloating potentials and enhancing qualities of digestion and palatability in animals feeding on it. The staggering progresses that are being currently made in legumes such as alfalfa will have important implications in the improvement of forage crops in the *Omic*s generation. Such studies and thoughts are clearly indicating towards the shift in the traditional opposition to GM crops. In several parts of the globe such Brazil, India, Iran, China, USA and Canada are increasing acreage under GM crops and indicating towards the wind of change in the global context of GM food and feed crops.

Conclusions and future directions

Forage production has come a long way from its initial importance of just sustaining the cattle, livestock and dairy industries. Forage in its true form is an "*industry in itself*" and Canada has been maintaining its' leadership position as *global giant* in sustainable forage production. It is not just catering to the Canadian agro-industries as mentioned above but is also participating in global trade and commerce earning the much needed foreign exchange for Canada and is also helping associated agro-industries on foreign shores to depend on reliable high quantity and quality of animal food and feed products. Slowly over the years from a modest beginning it has been turned into one of the major income and employment source for Canadians. A much of this has been achieved by the hard work and planning of dedicated illustrious plant breeders, crop scientists, soil scientists, agriculturists and agronomists. This is what we represent as the *Classical Age* of forage research or the *First Generation* forage research. Looking past beyond this era has come modernization in farming practices and extensive mechanization, innovation of new technologies in related disciplines of Chemical, Agricultural and Plant Sciences. With depleting environmental and ecological crises, Sustainable Agriculture has been turned into a buzzword and an important player in the free market economy concept of traditional Agricultural Economics. All these have tremendously impacted agricultural production and hence forage production. With the surge of Molecular Biological and Plant Biotechnological tool kits surging global crop development laboratories in an exponential manner such as recombinant DNA technology, molecular breeding, marker assisted selection, physical and molecular mapping, genetic engineering, RNAi technology, epigenetics and several other related developments we have reached the *Second Generation* or *High Technology Era* of forage research (Fig 3). Because all these technological developments going around different crop species include forage species or will soon be including them. However, with the availability of the human, Arabidopsis and rice genomics data being easily available in public domain warranties further research progress in forage crops too as in other plant species. Probably the complete genomic sequence of the *forage queen*

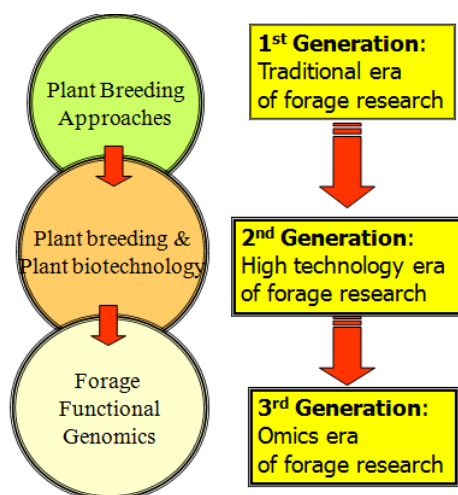


Fig 3. Transitional pattern of modern forage research.

alfalfa is not a distant, far-fetched goal any more. It will be coming today or tomorrow and will certainly mark a big or in other words epic change to the ways and traditions of forage production and development globally. The breeders working in association with plant biotechnologists and genetic engineers will have a much better grasp in playing with a wide range of genes to ensure higher productivity, better disease and abiotic stress resistances and faster maturation of the crop species. With the rapid development of biofuel industry forages will certainly play an important role in catering to such sister industries with rapidly growing cheap biomass for optimal biofuel production. We call this future forage research as the *Third Generation* of Forage research and development or the *Omics Era* in forage research (Fig 3). In addition to genomics, proteomics, metabolomics and transcriptomics will also ensure secure space and strata in the future forage research priorities.

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