PLENARY SESSION 2

Induced Mutations in the Genomics Era: New Opportunities and Challenges

Functional Genomic Analysis of Systemic Cell Division Regulation in Legumes

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Abstract

Legumes develop root nodules from pluripotent stem cells in the root pericycle in response to mitogenic activation by a decorated chitin-like nodulation factor synthesized in *Rhizobium* bacteria. The soybean genes encoding the receptor for such signals were cloned using map-based cloning approaches. Pluripotent cells in the root pericycle and the outer or inner cortex undergo repeated cell divisions to initiate a composite nodule primordium that develops to a functional nitrogen-fixing nodule. The process itself is autoregulated, leading to the characteristic nodulation of the upper root system. Autoregulation of nodulation (AON) in all legumes is controlled in part by a leucine-rich repeat receptor kinase gene (*GmNARK*). Mutations of *GmNARK*, and its other legume orthologues, result in abundant nodulation caused by the loss of a yet-undefined negative nodulation repressor system. AON receptor kinases are involved in perception of a long distance, root-derived signal, to negatively control nodule proliferation. *GmNARK* and *LjHAR1* are expressed in phloem parenchyma. GmNARK kinase domain interacts with Kinase Associated Protein Phosphatase (KAPP). NARK gene expression did not mirror biological NARK activity in nodulation control, as q-RT-PCR in soybean revealed high *NARK* expression in roots, root tips, leaves, petioles, stems and hypocotyls, while shoot and root apical meristems were devoid of *NARK* RNA. High through-put transcript analysis in soybean leaf and root indicated that major genes involved in JA synthesis or response are preferentially down-regulated in leaf but not root of wild type, but not NARK mutants, suggesting that AON signaling may in part be controlled by events relating to hormone metabolism. Ethylene and abscisic acid insensitive mutants of *L. japonicus* are described.

Nodulation in legumes has significance to global economies and ecologies, as the nitrogen input into the biosphere allows food, feed and biofuel production without the inherent costs associated with nitrogen fertilization [1]. Nodulation involves the production of a new organ capable of nitrogen fixation [2] and as such is an excellent system to study plant – microbe interaction, plant development, long distance signaling and functional genomics of stem cell proliferation [3, 4].

Concerted international effort over the last 20 years, using a combination of induced mutagenesis followed by gene discovery (forward genetics), and molecular/biochemical approaches revealed a complex developmental pathway that 'loans' genetic programs from various sources and orchestrates these into a novel contribution. We report our laboratory's contribution to the present analysis in the field.

Induced Mutants Facilitate Gene Isolation

In the 1980s we initiated targeted EMS and fast neutron mutagenesis of legumes (soybean; see [5, 6, 7, 8]) that led to the discovery of non-nodulation as well as supernodulation mutants. All alleles behave as loss-of-function mutations. Extensive characterization of both mutant classes was conducted [9, 10], revealing that NF is perceived by a LysM receptor kinase complex encoded by the *GmNFR1* and *GmNFR5* genes of soybean. Both genes, if mutated, lead to nodulation. Orthologous copies are found in other legumes, such as *Lotus japonicus*, *Medicago truncatula* and pea. In soybean, both receptor genes are duplicated (as are most soybean genes), owing to an ancestral genome duplication. Despite the duplicated genome, it was possible to isolate recessive, loss-of-function mutants because detailed genomic analysis revealed that the duplicated copies had functionally diverged. For example, the soybean *GmNFR5* gene exists as an α and β copy, with *GmNFR5β* being non-functional in many soybean cultivars because of the insertion of a transposable element (A. Indrasumunar, UQ, PhD dissertation; 2008). Other soybean nodulation related genes such as *GmNORK* (needed for both nodulation and mycorrhizal symbioses), *GmKAPP* (encoding kinase associated protein phosphatase; [11]) and *GmPOL* (poltergeist; another protein phosphatase) duplicated in an apparently fully functional fashion, suggesting that mutant phenotypes would be undetectable.

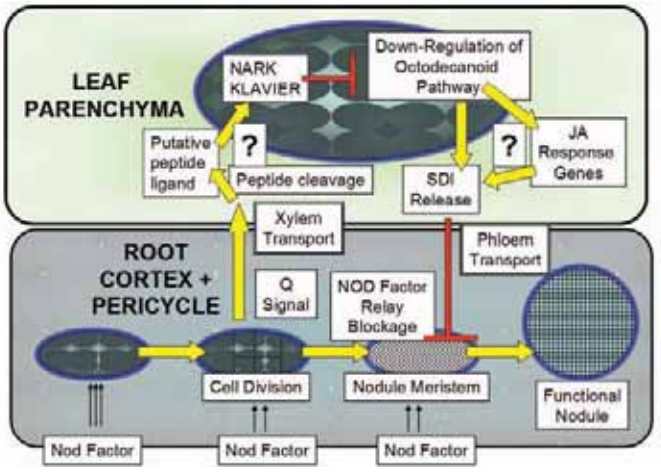

Figure 1 Model of autoregulation of nodulation (AON) as in soybean. Several points of uncertainty exist. SDI (shoot-derived inhibitor) and Q need to be determined; target stages are uncertain though cell division is blocked in soybean. Blockage in alfalfa and pea is earlier with no infection threads. Involvement of different root tissue lineage in this process is still unknown.

Mutants were also isolated for the internal regulation of nodulation, called AON [3, 5, 6, 12]. Again, similar mutants were later found in other legume species, confirming that the underlying genetic and biochemical mechanisms are similar if not identical. Super- or hyper-nodulation mutants exist in *L. japonicus*, *M. truncatula*, pea, and common bean [10, 13]. In most cases, supernodulation is associated with pleiotropic effects, such as nitrate tolerance to nodulation inhibition, altered root growth, and plant growth reductions [14, 15, 16]. Interestingly, NARK mutants also lack autoregulation of mycorrhization [17].

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Using positional cloning approaches based on detailed molecular maps, and BAC clone analysis, high throughput DNA sequencing and candidate gene approaches, the identity of mutated genes was revealed in many cases. Commonly, a LRR receptor kinase related in structure to the *Arabidopsis thaliana* CLAVATA1 gene product is mutated to lead to a supernodulation phenotype [18, 19, 20, 21,22]. Since reciprocal grafting clearly demonstrated that AON is controlled by the *GmNARK* or *LjHAR1* genotype of the shoot [12, 23], and that suppression of nodule number occurs subject to effective *Rhizobium/Bradyrhizobium* infection of the root, it was proposed that a root-derived nodulation signal (termed Q; [24]) migrates to the leaf where GmNARK converts the signal into a nodulation repressor (termed SDI; see **Fig. 1**.; [25, 26]).

Supernodulation mutants exist that do not fit directly into the NARK paradigm (such as *Psnod3; Mtrdn1*; *LjASTRAY* [27] and *LjKLAVIER* [28]. The first two, possibly orthologues, are root controlled and may be involved in the sending of the Q signal; the latter two function in the shoot, though their direct function, despite gene characterization (ASTRAY encodes a transcription factor, and KLAVIER a LRR RK) are characterised by changes in vascular development, flowering time, and root gravitropism.

Identification of Direct GmNARK Kinase Domain Interacting Protein Phosphatase

The putative catalytic domain of GmNARK was expressed and purified as a maltose-binding or a glutathione S-transferase-fusion protein in *Escherichia coli* [11]. The recombinant NARK proteins showed autophosphorylation activity *in vitro*. Several regions of the NARK kinase domain were shown by mass spectrometry to possess phospho-residues. The kinase-inactive protein K724E failed to autophosphorylate, as did three other proteins corresponding to phenotypically detected mutants defective in whole plant autoregulation of nodulation (AON). A wild-type NARK fusion protein transphosphorylates a kinase-inactive mutant NARK fusion protein, suggesting that it is capable of intermolecular autophosphorylation *in vitro*. In addition, Ser-861 and Thr-963 in the NARK kinase catalytic domain were identified as phosphorylation sites through site-directed mutagenesis. The genes coding for the kinase-associated protein phosphatases KAPP1 and KAPP2, two putative interacting components of NARK, were isolated. NARK phosphorylated recombinant KAPP proteins *in vitro*. Autophosphorylated NARK was, in turn, dephosphorylated by both KAPP1 and KAPP2. Our results suggest a model for the signal transduction pathway involving NARK in nodule development control.

Characterization of the *GmNARK* and *LjHAR1* Promoters

Based on qRT-PCR, *GmNARK* is expressed to varying levels throughout the plant; the transcript was detected at high levels in mature leaves and roots but to a lesser extent in young leaves, shoot tips, and nodules [29]. The transcript level was not significantly affected by *Bradyrhizobium japonicum* during the first week following inoculation. In addition, the activities of the promoters of *GmNARK* and *Lotus japonicus HAR1*, driving a β-glucuronidase (*GUSPlus*) reporter gene, were examined in stably transformed *L. japonicus* and transgenic hairy roots of soybean. Histochemical GUS activity in *L. japonicus* plants carrying either a 1.7-kb *GmNARK*pr::*GUS* or 2.0-kb *LjHAR1*pr::*GUS* construct was clearly localized to living cells within vascular bundles, especially phloem cells in leaves, stems, roots, and nodules. Phloem-specific expression was also detected in soybean hairy roots carrying these constructs [30]. Deletion analysis located a region controlling phloem-specific expression to a DNA sequence between 908 bp and 1.7 kb upstream of the translation start site of *GmNARK*. Regulatory elements required for the transcription of these orthologous genes are conserved. Moreover, rapid amplification of 5' cDNA ends (5' rapid amplification of cDNA ends) revealed two major transcripts of *GmNARK* potentially originating from two TATA boxes. Further analysis of the *GmNARK* promoter has confirmed that these two TATA boxes are functional.

Search for Signal Molecules in Autoregulation of Nodulation

Bioassays based on nodule numbers and the expression of marker genes have been developed to identify Q in soybean xylem sap and SDI in leaf and phloem extracts. These bioassays will aid in confirming the presence of Q and SDI in samples following purification and fractionation procedures. Such findings will provide a more complete understanding of AON and may lead to the chemical identification of Q and SDI.

To characterize SDI, we developed a short-term nodulation-based bioassay involving feeding aqueous leaf extracts into the petioles of *nts1007* supernodulation mutant plants. Leaf extracts from 18-day-old *Bradyrhizobium japonicum* root-inoculated WT plants suppressed the supernodulation phenotype of *nts1007* plants to near wild-type levels. In contrast, feeding extracts from mutant leaf into wild type plants had no effect on nodule numbers. SDI suppression was *Bradyrhizobium*-inoculation dependent, required GmNARK activity, and was heat-, proteinase K- and RNase A-resistant. Extracts were stable and dilutable [31]. Molecular sieving confirmed SDI to be a small compound, with a molecular weight of less than 1,000 daltons. Feeding leaf extracts from *Sinorhizobium*-inoculated *Medicago truncatula* WT plants also suppressed *nts1007* nodule numbers, while extracts from mutant plants altered in *MtSUNN*, orthologous to *GmNARK*, failed to suppress, suggesting the AON molecular mechanism to be widely conserved amongst legumes.

Legumes encode several LRR-RLK linked to the process of root nodule formation, the ligands of which are unknown. To identify ligands for these receptors, Oelkers et al [32] used a combination of profile hidden Markov models and position-specific iterative BLAST, allowing the detection of new members of the CLV3/ESR (CLE) protein family from publicly available sequence databases.

They identified 114 new members of the CLE protein family from various plant species, as well as five protein sequences containing multiple CLE domains, and were able to cluster the CLE domain proteins into 13 distinct groups based on their pairwise similarities in the primary CLE motif. In addition, we identified secondary motifs that coincide with our sequence clusters. The groupings based on the CLE motifs correlate with known biological functions of CLE signaling peptides and are analogous to groupings based on phylogenetic analysis and ectopic overexpression studies. The biological function of two of the predicted CLE signaling peptides was tested in the legume *Medicago truncatula*. These peptides inhibit the activity of the root apical and lateral root meristems in a manner consistent with our functional predictions based on other CLE signaling peptides clustering in the same groups.

Hormonal Sensitivity Mutants in *Lotus japonicus* to Study Nodulation

Using transgenics, a causal relationship between ethylene insensitivity of seedlings and mature legume plants was demonstrated [33], the level of ethylene receptor gene expression, lateral root growth, and *Mesorhizobium loti* inducing nodule initiation. *Lotus japonicus* plants expressing the dominant *ETR1-1* allele from *Arabidopsis thaliana* (*AtETR1-1*) encoding a mutated ethylene receptor were ethylene insensitive as judged by the lack of "Triple Response," and their continued ability to grow and nodulate in the presence of inhibitory concentrations of ACC (1-aminocyclopropane-1-carboxylic acid; an ethylene precursor). Independent transgenic lines varied in their levels of insensitivity that correlated directly to the number of nodulation events after *M. loti* infection and the level of *AtETR1-1* mRNA. Transgenic plants with high insensitivity to ACC had significantly fewer lateral roots and exhibited increased nodulation while showing no altered nitrate sensitivity or lack of systemic autoregulation. While ACC insensitive shoot growth and

nodulation were observed in transformants, primary root growth was still inhibited similarly to wild type. Increased nodulation was caused by increased infection success and a seven-fold increase of the permissive radial zone for initiating nodule foci. The study demonstrates multiple roles for ethylene and cytokinins in nodule initiation by influencing root cell infections and radial positioning [34], independent of autoregulation and nitrate inhibition of nodulation.

An ABA insensitive mutant *Beyma* was isolated in *Lotus japonicus* MG-20 from an EMS mutagenesis population using root growth inhibition to applied ABA as the screening criterion [35]. The stable mutant that segregates as a dominant Mendelian mutation is insensitive to ABA induced inhibition of germination, vegetative growth, stomatal opening as well as nodulation. Tissue ABA levels were normal, suggesting a sensitivity rather than biosynthesis mutation. It is slow-growing (50-70 % of MG-20) and has a near-constitutive wilty phenotype associated with its inability to regulate stomatal opening. Whilst showing a wide range of ABA insensitive phenotypes, *Beyma* did not show alteration of nodule number control, as in the absence of added ABA, the number and patterning (but not size) of nodules formed in the mutant was similar to that of MG-20. Split root experiments on MG-20 showed that application of ABA on one side of the root inhibited nodulation locally but not systemically. We propose that ABA is not involved directly in systemic autoregulation of nodulation (AON).

Transcript Profiling of Soybean Leaves Reveals Novel Candidate Biochemical Networks for AON Function

Kinkema and Gresshoff [36] used transcriptional profiling to identify potential downstream signals of GmNARK. These studies revealed that GmNARK-mediated signaling controls the expression of genes involved in the JA pathway. Genes encoding the key enzymes controlling JA biosynthesis as well as JA-response genes were regulated systemically, but not locally, by root inoculation with Bradyrhizobium japonicum. This systemic regulation was abolished in Gmnark mutant plants, indicating that their expression was specifically controlled by signaling events associated with this receptor kinase. These results indicate that the receptor-mediated regulation of JA signaling plays an important role in the AON signal transduction pathway. A second class of genes was identified that were controlled by GmNARK in a rhizobia-independent manner. These candidates provide insight on additional, non-symbiotic signaling pathways that are likely regulated by GmNARK, such as those involved in root growth and defense [37]. The discovery of downstream components of the GmNARK receptor kinase advances our understanding of the systemic control of nodule development and its association with other signaling networks.

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Metabolite Profiling of Induced Mutants of Rice and Soybean

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Abstract

The low phytic acid (*lpa*) rice (*Os-lpa*-XS110-1, *Os-lpa*-XS110-2) and soybean (*Gm-lpa*-TW-75-1, *Gm-lpa*-ZC-2) mutants generated by γ-irradiation were studied, aimed at comparing these mutants to the corresponding wild-types by means of metabolite profiling based on capillary gas chromatography/mass spectrometry. The usefulness of this approach to assist in the elucidation of the types of mutation resulting in reduced contents of phytic acid should be explored. Metabolite profiling aspires to provide a comprehensive picture of the metabolites present in biological systems. It aims at extracting, detecting, identifying, and quantifying a broad spectrum of compounds in a single sample, to provide a deeper insight into complex biological systems. The extraction and fractionation method used allowed a comprehensive coverage of a broad spectrum of low molecular weight metabolites ranging from lipophilic (fatty acids methyl esters, hydrocarbons, free fatty acids, sterols, tocopherols) to hydrophilic (sugars, sugar alcohols, organic acids, amino acids) compounds. For rice, considerable amounts of the peaks detected were statistically significantly different between wild-types and *lpa* mutants grown in the same field trial. However, only a few of these differences could be consistently observed in all analyzed field trials, indicating a strong influence of the biological variability. Metabolites consistently shown to be significantly different between wild-type and *lpa* rice mutants, were found to be closely related to the biogenetic pathways leading to phytic acid. This allowed a prediction of the mutation targets for the *lpa* rice mutants in the biosynthetic pathway of phytic acid. Similar effects, i.e. statistically significantly different levels of metabolites closely related to the biosynthesis of phytic acid, were consistently observed for soybean.

Introduction

Myo-inositol 1,2,3,4,5,6-hexa*kis*phosphate (phytic acid or Ins(1,2,3,4,5,6)P_6) is the major storage form of phosphorus in plants [1]. Approximately, 65% to 85 % of total phosphorus in mature plant seeds is found in this compound [2,3].

The first step in the biosynthesis of phytic acid represents the conversion of D-glucose 6-phosphate to 1D-*myo*-inositol 3-phosphate (Ins(3)P_1) catalyzed by 1D-*myo*-inositol 3-phosphate synthase (MIPS) [4,5]. The subsequent steps leading to phytic acid are not fully clarified. Stepwise phosphorylation of Ins(3)P_1 to phytic acid seems plausible, especially as a phosphoinositol kinase which phosphorylates $InsP_1$, $InsP_2$, $InsP_3$, $InsP_4$ and $InsP_5$ to the next higher homolog has been identified in mung bean [6]. In addition to Ins(3)P_1, free *myo*-inositol formed through dephosphorylation of Ins(3)P_1 has been discussed as an intermediate in the biosynthesis of phytic acid. A *myo*-inositol kinase (MIK) has been isolated from maize which phosphorylates *myo*-inositol but not Ins(3)P_1. Mutation of the gene encoding MIK resulted in a significant decrease in phytic acid content [7].

Phytic acid represents an anti-nutrient in food and feed. It limits the bioavailability of minerals such as iron, zinc, calcium and selenium by formation of indigestible chelates [8,9]. In addition, phytic acid is poorly degraded in the digestive system of humans and non-ruminants [10]. Thus, the phytic acid phosphorus is not bioavailable. Animal feed producers and farmers must therefore add phosphate to feed to ensure its nutritional quality. Moreover, excreted phytic acid in manure is degraded by natural soil microorganisms releasing phosphate, which contributes to eutrophication of water [11].

Various efforts have been made to breed crop varieties low in phytic acid content. Transformation of the MIPS gene in antisense orientation into rice plants resulted in a significant increase in inorganic phosphate, which indicates a molar-equivalent decrease in phytic acid [12]. In addition to this targeted molecular approach, mutation breeding has been successfully applied to generate low phytic acid (*lpa*) crops. *Lpa* mutants have been generated for maize [13-15], barley [16], rice [17,18], soybean [19,20] and wheat [21]. In order to identify mutation targets responsible for decreased phytic acid levels genetic approaches and targeted analysis of metabolites involved in the biosynthesis of phytic acid have been applied. In a soybean *lpa* mutant a single base change in the MIPS gene leading to decreased enzyme activity was detected by gene sequencing [20]. Targeted analysis of inositol phosphates in barley *lpa* mutants revealed that reduction in phytic acid was accompanied by an increase in $InsP_3$, $InsP_4$ and $InsP_5$, which suggested a lesion in the phosphorylation steps rather than in the MIPS gene [16].

In addition to genetic approaches and targeted analysis of individual compounds, metabolite-profiling techniques have been proposed as useful tools for plant functional genomics [22]. Metabolite profiling aspires to provide a comprehensive picture on the metabolites present in biological systems. It aims at extracting, detecting, identifying, and quantifying a broad spectrum of compounds in a single sample to provide a deeper insight into complex biological systems. Moreover, metabolite-profiling techniques have been proposed as valuable tools for the detection of unintended effects caused by genetic engineering of food crops [23]. In case of new plant varieties developed with traditional techniques, application of metabolite profiling for the assessment of the safety of these crops has also been suggested [24,25].

The objective of this study was to compare *lpa* mutants of rice (*Os-lpa*-XS110-1, *Os-lpa*-XS110-2) and soybean (*Gm-lpa*-TW751, *Gm-lpa*-ZC-2) with their corresponding rice (Xiushui 110) and soybean wild-types (Taiwan 75, Zhechun No. 3) on the basis of metabolite profiling. Recently, first attempts to apply metabolomic analysis to low phytic acid mutants of maize [26] and rice [27] have been reported.

Description of the metabolite profiling procedure

Metabolite profiling was performed according to the extraction and fractionation scheme shown in Figure 1 [28]. The experimental procedure has

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been described in detail [27]. Briefly, lipids and polar compounds were consecutively extracted from the freeze-dried rice and soybean flour. Lipids were transesterified in methanol and subsequently separated by solid phase extraction into a fraction containing fatty acid methyl esters (FAME) and hydrocarbons (fraction I, **Fig. 2A**) and a fraction containing minor lipids, e.g. free fatty acids, fatty alcohols and sterols (fraction II, **Fig. 2B**). Selective hydrolysis of silylated derivatives was applied to separate the polar extract into a fraction containing silylated sugars and sugar alcohols (fraction III, **Fig. 2C**) and a fraction containing inorganic and organic acids, amino acids and amines (fraction IV, **Fig. 2D**). The four fractions obtained were analyzed by capillary gas chromatography (GC-FID and GC-MS).

Peak heights and corresponding retention times were exported to Chrom*pare*, a software tool developed for comparative analysis of metabolite profiling data [29] (www.chrompare.com). Chrom*pare* automatically corrects retention time shifts on the basis of retention time standards and standardizes peak heights on the basis of internal standards added before the fractionation process. Comparison of metabolite profiles is performed by comparison of mean standardized peak heights based on triplicate analysis. Means are considered as statistically significant different if their confidence intervals ($p < 0.05$) are not overlapping. The magnitude of difference is calculated and peaks observed in only one sample are reported as additional/missing peaks.

Metabolite profiling of low phytic acid (*lpa*) mutants

Low phytic acid (*lpa*) rice mutants

For investigation of the overall variance between *lpa* rice mutants *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2, GC-FID metabolite profiling data were analyzed by means of principal component analysis (PCA). PCA plots containing GC-FID data from fractions III and IV are shown in **Figure 3**.

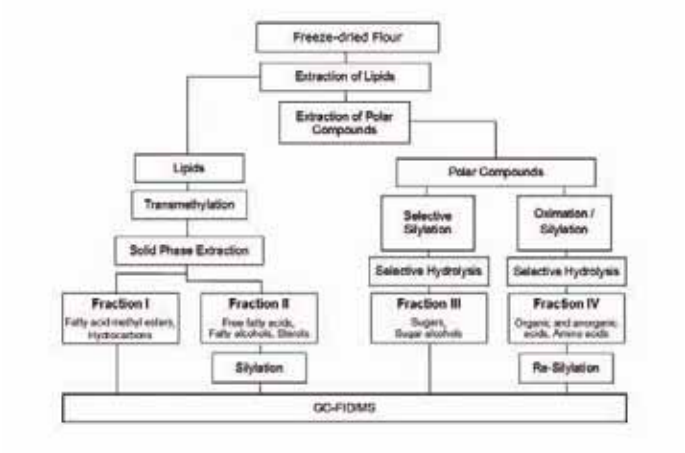

Figure 1 Extraction and fractionation of freeze-dried rice and soybean flour, according to [28].

A clear separation of *Os-lpa*-XS110-1 from Xiushui110 was observed, whereas no differentiation between *Os-lpa*-XS110-2 and the wild-type could be achieved. This indicated different types of mutations for the two *lpa* rice mutants.

Results obtained by comparative metabolite profiling of the wild-type Xiushui110 and the two *lpa* rice mutants are shown in Table 1. For each field trial the sum of peaks included for comparison was more than 100. On average, 34% (*Os-lpa*-XS110-1) and 42% (*Os-lpa*-XS110-2) of the peaks included were statistically significantly different between the wild-type and the mutants [27].

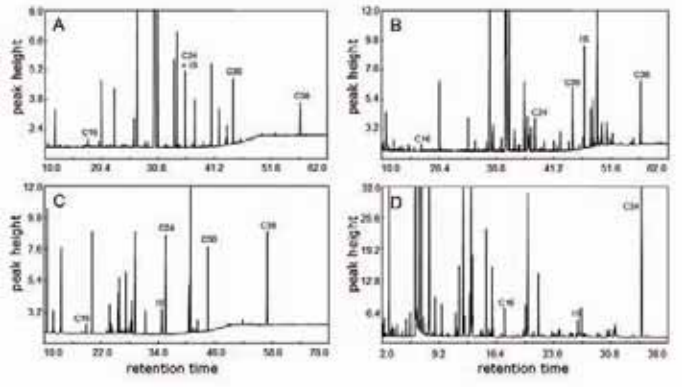

Figure 2 GC-FID chromatograms of fraction I (A), II (B), III (C) and IV (D) obtained from rice. IS: internal standards tetradecane (A), 5α-cholestane-3β-ol (B), phenyl-β-D-glucopyranoside (C), p-chloro-L-phenylalanine (D). C16, C24, C30, C38: retention time standards hexadecane, tetracosane, triacontane and octatriacontane.

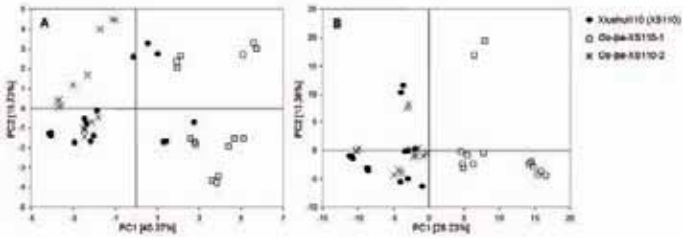

Figure 3 Principal component analysis (PCA) of standardized GC-FID metabolite profiling data from fraction III (A) and IV (B) of rice wild-type Xiushui110 and the two low phytic acid mutants *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2.

Table 1. Peak-based comparison of chromatograms obtained by metabolite profiling of fractions I-IV of wild-type rice Xiushui110 (XS110) and low phytic acid mutant lines *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2

	Field trial								
	Hainan		Jiaxing		Hangzhou 1		Hangzhou 2		
wild-type vs. lpa mutant	total[a]	diff.[b]	total	diff.	total	diff.	total	diff.	consistent differences[c]
XS110 vs. *Os-lpa*-XS110-1	123	38	119	38	115	45	108	38	4
XS110 vs. *Os-lpa*-XS110-2	118	27	116	63	113	35	108	67	2

[a] Number of peaks included for comparison
[b] Number of peaks statistically significant different between wild-type and mutant ($p < 0.05$)
[c] Number of peaks statistically significant different between wild-type and mutant at all four field trials

Although the number of peaks statistically significantly different at one field trial was relatively high, only a few of these differences could be consistently observed at all four field trials. Identification of these consistent differences revealed increased contents of phosphate in both mutant lines. Levels of galactose, raffinose and *myo*-inositol were consistently increased in *Os-lpa*-XS110, whereas the level of *myo*-inositol was decreased in *Os-lpa*-XS110-2. Further, the level of 24-methylenecycloartanol (24-MCA) was consistently increased in this mutant. Changes in mean standardized peak heights of phosphate, *myo*-inositol and raffinose for both *lpa* mutants compared to wild-type Xiushui110 are shown in **Figure 4**.

Except for 24-MCA, metabolites shown to be consistently statistically significantly different between wild-type and *lpa* rice mutants, were found to be closely related to the biogenetic pathways leading to phytic acid (Figure 5). Consideration of these metabolic changes in the light of the routes involved in the biosynthesis of phytic acid indicated a disturbance in the early biosynthetic pathway of phytic acid in *Os-lpa*-XS110-2 and a mutation event affecting phosphorylation of *myo*-inositol in *Os-lpa*-XS110-1 [27]. The metabolite profiling-based prediction for *Os-lpa*-XS110-1 was in accordance with molecular mapping results, which placed the *lpa* mutation on a site very close to the locus that encodes the putative *myo*-inositol kinase (MIK) gene in rice [18].

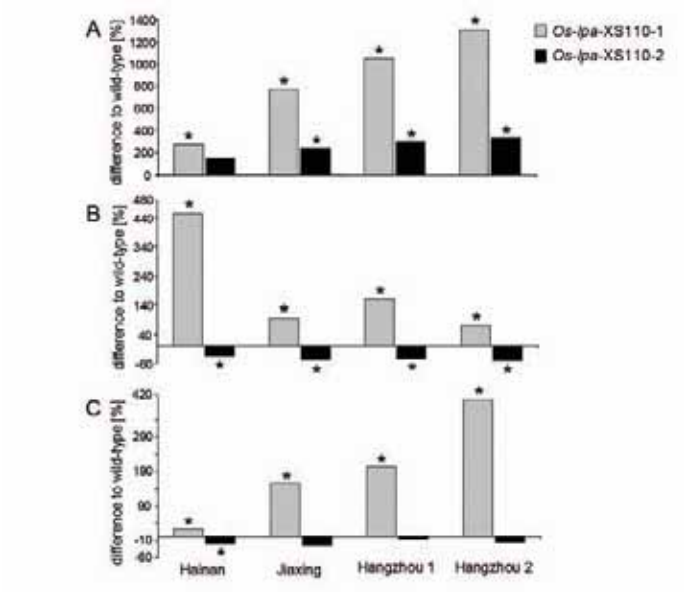

Figure 4 Differences in mean standardized peak heights (triplicate analysis) of phosphate **(A)**, *myo*-inositol **(B)** and raffinose **(C)** between wild-type Xiushui110 and the two mutant lines *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 grown in four field trials. An asterisk indicates a statistically significant difference from the wild-type ($p < 0.05$).

Low phytic acid (*lpa*) soybean mutants.

Results obtained by comparative metabolite profiling of the wild-type soybeans Taiwan75 and Zhechun No. 3 and the two corresponding *lpa* mutants *Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2 are shown in **Table 2**.

Table 2. Peak-based comparison of chromatograms obtained by metabolite profiling of fractions I-IV of wild-type soybeans (Taiwan 75, Zhechun No.3) and low phytic acid mutant lines *Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2

	Field trial								
	Hainan 4/05		Hainan 05/06		Hangzhou s05		Hangzhou a05		
wild-type vs. *lpa* mutant	total[a]	diff.[b]	total	diff.	total	diff.	total	diff.	consistent differences[c]
TW75 vs. *Gm-lpa*-TW75-1	-[d]		168	69	181	65	165	70	23
Zh. No.3 vs. *Gm-lpa*-ZC-2	166	27	164	55	157	13	167	43	3

[a] Number of peaks included for comparison
[b] Number of peaks statistically significant different between wild-type and mutant ($p < 0.05$)
[c] Number of peaks statistically significant different between wild-type and mutant at all fourfield trials
[d] Samples not available

For each field trial the sum of peaks included for comparison was more than 150. On average, 40% (*Gm-lpa*-TW75-1) and 21% (*Gm-lpa*-ZC-2) of the peaks included were statistically significantly different between the wild-types and the mutants.

Again, a majority of the metabolites shown to be consistently statistically significantly different between wild-types and *lpa* soybean mutants, were found to be closely related to the biogenetic pathways leading to phytic acid (**Figure 5**). For example, levels of *myo*-inositol were decreased in *Gm-lpa*-TW75-1 compared to Taiwan 75, but increased in *Gm-lpa*-ZC-2 compared to Zhechun No. 3. Changes observed on the basis of metabolite profiling indicate a mutation in the 1D-*myo*-inositol 3-phosphate synthase (MIPS) (**Figure 5**). This mutation target was also postulated on the basis of data from molecular mapping [30].

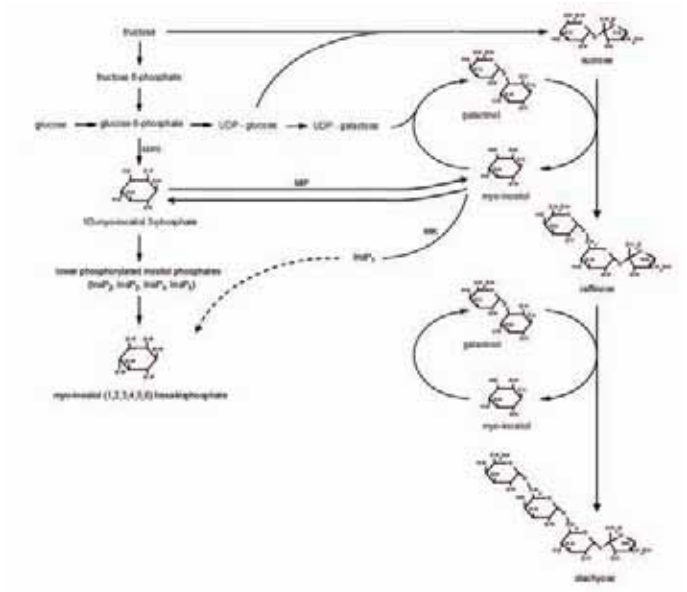

Figure 5 Plant biosynthetic patways leading to phytic acid, raffinose and stachyose. The dotted line indicates a not fully clarified pathway. MIPS: 1D-*myo*-inositol 2 phosphate, MIP: *myo*-inositol monophosphatase, MIK: *myo*-inositol kinase.

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Genomics Meets Induced Mutations in Citrus: Identification of Deleted Genes Through Comparative Genomic Hybridization

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Abstract

We report on the use of genomic approaches to identify pivotal genes in induced citrus mutants. Citrus is the most economically important fruit crop in the world and Spain is the first fresh citrus producer. The survival of the citrus industry is critically dependent on genetically superior cultivars but improvements in fruit quality traits through traditional techniques are extremely difficult due to the unusual combination of biological characteristics of citrus. Genomic science, however, holds promise of improvements in breeding. In this work, we reported the successful identification of genes included in hemizygous deletions induced by fast neutron irradiation on *Citrus clementina.* Microarray-based CGH was used to identify underrepresented genes in a citrus mutant that shows color break delay. Subsequent confirmation of gene doses through quantitative PCR and comparison of best hits of putative deleted citrus genes against annotated genomes from other eudicots, specially poplar, enabled the prediction that these genes were clustered into a 700 kb fragment. The availability of *Citrus* BAC end sequences helped to draw a partial physical map of the deletion. Furthermore, gene content and order in the deleted segment was established by PCR location of gene hits on the physical map. Finally, a lower chlorophyll a/b ratio was found in green tissues from the mutant, an observation that can be related to the hemizygous deletion of a ClpC-like gene, coding a putative subunit of a multifunctional protease complex located into the chloroplast. Analysis of gene content and order inside this *Citrus* deletion led to the conclusion that microsynteny and local gene colinearity with *Populus trichocarpa* were higher than with the phylogenetically closer *Arabidopsis thaliana* genome. In conclusion, a combined strategy including genomics tools and induced citrus mutations has been proved to be a successful approach to identify genes with major roles in citrus fruit development.

Introduction

Citrus, one the most important fruit crops worldwide, are woody, perennial trees requiring a juvenility period of several years and are frequently parthenocarpic and sexually self-incompatible [1]. These conditions considerably impair traditional breeding, while current alternative methods for variety improvement such as marker-assisted selection, somatic hybridization or mutation breeding also are of limited use mostly because the *Citrus* genome is scarcely characterized.

In recent years, *Citrus* has been the target of several genomic developments including large EST collections [3,4], cDNA and oligonucleotide-based microarrays [5-8], BAC libraries and BAC end sequencing (BES). Genomic technology, including methods to rapidly identify and manipulate genes of agricultural interest, holds promise of improvements in several areas that may be difficult through traditional approaches. However, functional studies, i.e. genetic transformation and the capability to perform reverse genetic analyses, are also considerably impaired. In citrus, high throughput transgenic programs such as the generation of RNA interference knockouts, activation tagging through enhancer elements, gene-trap T-DNA insertions, or transposon tagging systems have not yet been developed. Since no efficient tagging or insertional procedures are available in these species, other gene disruption methods including strategies based on genome-wide mutagenesis such as TILLING and fast neutron mutagenesis have been initiated. These approaches are non-transgenic and may have particular interest for the industry where the debate on genetically modified organisms has restricted crop improvement. Both approaches, however, are of limited usefulness as strategies for reverse genetics because of the lack of knowledge on *Citrus* genomic sequence and the large amount of space required for mutant populations of a suitable size. ECOTILLING, however, on natural citrus variants and microarray-based detection of deletions on fast neutron citrus mutants in a more direct genetic strategy are apparently very straightforward approaches.

The main objective of this work was to identify deleted genes on a heterozygous genetic *Citrus* background, provided by fast neutron generated mutants, through array-Comparative Genomic Hybridization. In addition, we also explored the possibility of using comparative genomics with annotated dicot genomes assisted by BAC end sequencing for the generation of realistic partial physical maps of the deleted *Citrus* regions.

Materials and Methods

Plant material

Plant material from approximately six-year-old standard and 39B3 and 39E7 mutant lines of the clementine (*Citrus clementina* Hort. Ex Tan. cv. Clemenules) variety was used in this study. The 39B3 and 39E7 genotypes that showed altered patterns of color change of fruit peel were obtained through bud irradiation with fast neutrons and are expected to carry DNA deletion lesions in hemizygous dosage.

Array-CGH

The protocol was adapted from several published array-Comparative Genomic Hybridization (array-CGH) methods pursuing mainly the measurement of copy-number changes in human genomic DNA [9-11], and the study of large-scale genetic variation of the symbiotic bacteria *Sinorhizobium meliloti* [12].

Gene dosage measurements

Quantitative real-time PCR was performed on a LightCycler 2.0 instrument using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit following manufacturer's instructions.

Similarity searches

DNA sequences of *Citrus* unigenes containing positive array-CGH ESTs were used in online TBLASTX searches against genomic databases from the annotated genomes of *Arabidopsis thaliana* [13], *Populus trichocarpa* [14] and *Vitis vinifera* [15]. For each gene, the best hit was placed on a chromosomal map while the second and third hits were only positioned

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in the map if they were located closer than 250 kb to any other hit. Two 700 kb regions from chromosomes 12 and 15 from the *Populus* genome including homologous genes to 39B3 array-CGH positive unigenes, were used as queries in a BLASTN local search on a *Citrus* BAC end sequence database.

BAC isolation and analysis

DNA from *Citrus* BACs was isolated with the Rapid Plasmid Miniprep System (Marligen Biosciences, cat. nº 11453-016). Purified BACs were used as templates in PCR reactions in a total volume of 15 µl, including 0.2 mM dNTP, 2 mM $MgCl_2$, 0.5 µM of each primer, 0.38 units of Netzyme DNA polymerase (Molecular Netline Bioproducts) and 0.1 ng of BAC DNA. After an initial denaturing step for 5 min at 95°C, amplification was performed for 35 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C, followed by 5 min incubation at 72°C. The PCR product was subjected to 1.5% agarose DNA electrophoresis.

Results and Discussion

Procedure for the characterization of hemizygous deletions

The proposed procedure to identify deleted genes is illustrated in **Figure 1**. This method uses microarrays to hybridize genomic DNA extracted from the deletion mutants to render a list of underrepresented genes. The putative deleted genes are then validated through gene dosage evaluation by real-time PCR with specific primers. Deleted genes may in this way contribute to the identification of the molecular mechanisms underlying the observed phenotypes by means of a candidate gene approach, validated by physiological analyses or genetic transformation. In non-sequenced genomes or in plants with poor development of physical maps, further characterization of deletions at the structural level requires TBLASTX similarity searches against databases containing the sequence annotation of known eudicot genomes. Local physical maps of deletions are built allocating the deleted gene sequences and the syntenic genomic fragments from these eudicots into a BES database of the species of interest. Lastly, specific PCR on the array of BACs contributes to confirm gene content and order on the lineal structure of the deletions.

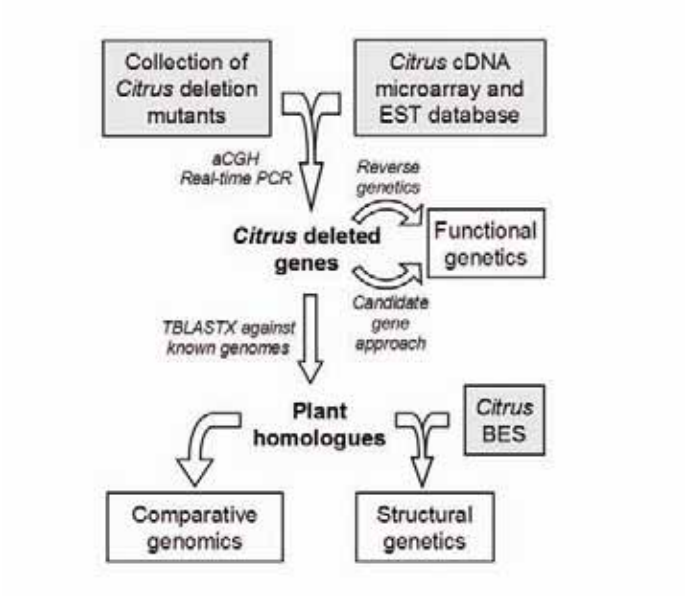

Figure 1 Schematic guidelines for the characterization of hemizygous deletion Citrus mutants. Arrows indicate successive steps of mutant characterization. Plant material and genomic resources are highlighted on grey boxes while gained knowledge on genetics and genomics are highlighted on white rectangles. Genes are shown in bold and methods and approaches in italics.

Identification of deleted alleles in 39B3 and 39E7 fast neutron mutants of Citrus clementina

Putative deleted genes in the mutants were first identified through an approach based on genomic hybridization (array-CGH) that exploited a recently developed *Citrus* microarray containing 21240 cDNAs [13,14]. The number of ESTs selected as putative candidates was 24 and 78 for mutants 39B3 and 39E7, respectively. Gene dosage of 39B3 candidates ranged from 0.50 to 0.60 when genomic DNA from the 39B3 and 39E7 genotypes was tested.

Clustering of homologues of Citrus deleted genes in the poplar genome

The homologous regions produced by the best TBLASTX hit of each of the *Citrus* candidate genes were located on the chromosome maps of *Arabidopsis*, poplar and grapevine [15]. **Figure 2** represents in detail chromosome mappings of the 39B3 mutation, which was subjected to further analyses. In *Populus,* most of the candidate genes mapped to two different genome regions of approximately 700 kb long in chromosomes 12 and 15, two duplicated chromosomes probably originated during the recent genome duplication event occurred in this species.

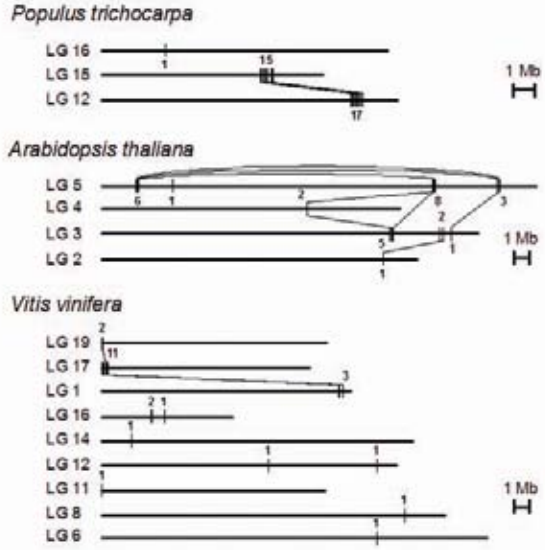

Figure 2 Chromosome mapping of poplar, Arabidopsis and grapevine homologues of the 39B3 Citrus deleted genes. The first TBLASTX hit for each *Citrus* deleted gene with an E value cut-off < 10-5 is represented on linkage groups (LG) from *Populus trichocarpa*, *Arabidopsis thaliana* and *Vitis vinifera*. Homologues of Citrus genes were grouped into clusters in each species when the distance between them was shorter than 250 kb. Second and third hits are only represented when they are located in a previously identified cluster, and in this case are linked to the first hit by a line. The value on each cluster indicates the hit number of the cluster.

Gene arrangement and partial physical map of the 39B3 deletion

The closer microsynteny observed between the 39B3 deletion and the two duplicated homologous regions in poplar enabled to predict gene structure by direct inference of gene position from the *Populus* sequences. Two DNA sequences covering 700 kb along the *Populus* chromosomes 12 and 15, containing the genes homologous to the *Citrus* deleted candidates, were BLASTed against available *Citrus* BAC end sequences. A partial physical map containing 13 BACs systematically named B1 to B13 was provided by standard PCR of BAC end amplicons against BAC templates and *in silico* search of overlapping antiparallel ends (**Figures 3A, 3B**). The above results indicated that the microsynteny between *Citrus* and *Populus* genomes was high enough to predict gene arrangement and to build a partial physical map of a *Citrus* genomic segment of about 700 kb, as inferred from the length of poplar homologous regions.

Overall, the data indicated that the *Populus* genome is a useful model for comparative genomics and that can be used to characterize hemizygous deletions in *Citrus*.

The *Citrus* 39B3 deletion shows higher local gene colinearity with *Populus* than with *Arabidopsis.*

We also mapped by PCR the 21 putative deleted genes on the physical map of **Figure 4A, 4B.** These results indeed confirm high local gene colinearity with poplar in the genomic region covered by 39B3 deletion.

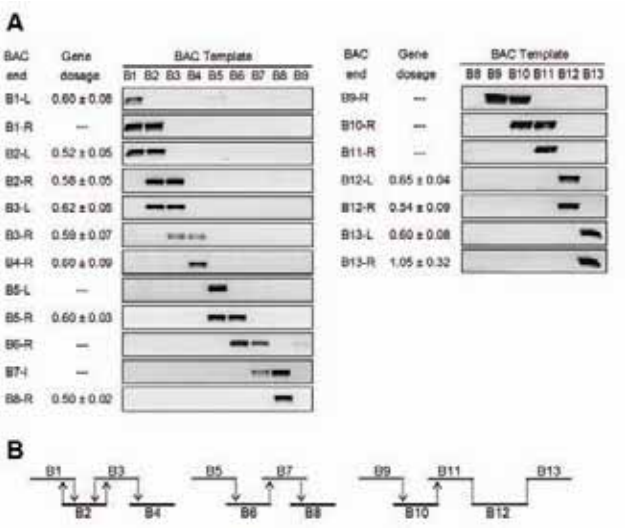

Figure 3 Local physical mapping of the Citrus 39B3 deletion. **(A)** Electrophoretic analysis of PCR products showing overlapping BACs. Purified BAC templates are distributed horizontally and divided in two panels. Primer pairs were designed from BAC end sequences containing non-repetitive DNA and named with the number of the BAC plus "-L" for left end and "-R" for right end according to the drawing orientation. B7-I primers amplify an internal sequence from B7 instead of an end. Gene dosage measurements for some of the primer pairs in the 39B3 genotype are shown on the left side of the electrophoretic images. **(B)** Physical map of the *Citrus* 39B3 deletion. Horizontal lines represent BACs, which are numbered from left to right. Vertical arrows show overlapping as inferred from PCR reactions and the head of the arrow indicates the BAC template. The vertical lines without arrow show connection of B11 with B12 by sequence of unigene aCL4690Contig1 and B12 with B13 by aCL1915Contig2 instead of a PCR reaction.

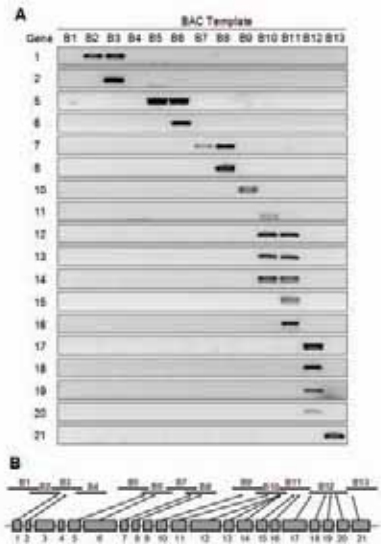

Figure 4 Gene arrangement on the physical map of the Citrus 39B3 deletion. **(A)** Primer pairs designed for the putative deleted genes included in the 39B3 deletion (**Table 2**) were utilized in PCR reactions on the BAC templates shown in **Table 3**. Genes are numbered and arranged vertically, on the left side of the electrophoretic image, and BAC templates are listed horizontally. **(B)** Citrus genes included in the 39B3 deletion and arranged as drawn previously but without indication of strand sense, are connected with arrows to the deletion physical map according to PCR results.

Taking together gene content and order conservation, it is inferred that in the studied DNA deleted segment there was higher gene colinearity with *Populus*, which diverged about 109 million years ago (Mya), than with *Arabidopsis*, splitting from the *Citrus* lineage about 87 Mya, despite gene colinearity is generally correlated with phylogenetic closeness. In papaya, BES alignment to the annotated genomes rendered higher gene colinearity with *Populus* than with *Arabidopsis*, although both *Arabidopsis* and papaya belong to the order Brassicales. In melon, microsynteny studies based on the sequence of two BACs also concluded that melon was closer to *Populus* than to *Arabidopsis* or *Medicago truncatula*. These observations may be explained by a differential genome evolutionary dynamics in poplar and *Arabidopsis* lineages. The more recent appraisals estimated that last whole genome duplications occurred not later than 60-65 Mya in *Populus* and around 24-40 Mya in *Arabidopsis* lineages. Despite the older poplar event, genome rearrangements involving gene loss and translocation following these duplications were much more frequent in *Arabidopsis* ancestors. Such a highly active genome dynamics probably caused the dispersion of genes and the subsequent reduction in synteny and gene colinearity with even related species. The different behavior of *Populus* and *Arabidopsis* ancestral genomes still deserves further explanation. It has been suggested that woody long-lived species like poplar trees may undergo a slower genome dynamics due to their juvenile period that delays sexual fecundation for several years and to the recurrent contribution of gametes from aged individuals of previous generations. In addition, species like *Arabidopsis thaliana* may have very active mechanisms for unequal or illegitimate recombination causing frequent chromosomal rearrangements such as translocations, insertions and deletions.

Chlorophyll *a/b* ratio is modified in 39B3 mutant

Among the deleted genes, one of the 39B3 hits validated by real-time quantitative PCR coding for a ClpC-like protein, may have certain relevance in the 39B3 phenotype. Plant ClpCs are ATP-binding proteins located in the stroma of chloroplasts, which have been found to be associated with the protein import machinery. In *Arabidopsis* a mutant impaired in *ClpC1* mRNA processing accumulated chlorophyllide *a* oxygenase protein (CAO), a key enzyme for the synthesis of chlorophyll *b* from chlorophyll *a*, leading to a reduced chlorophyll *a*/*b* ratio. Interestingly, 39B3 mutants also contained a lower ratio of chlorophyll *a*/*b* in young and old leaves and fruit exocarp.

Conclusions

In this study, we propose a procedure for the genetic characterization of genomic hemizygous deletions in mutants from plant species with non-sequenced genomes. The procedure is illustrated with the study of the 39B3 *Citrus clementina* deletion, generated by fast neutron bombardment. The proposed strategy utilizes several genomic resources such as array-Comparative Genomic Hybridization (array-CGH) technology, EST and BAC end sequencing databases and poplar genome annotation. The array-CGH results led to the conclusion that the 39B3 deletion removed at least 21 genes while a partial physical map of about 700 kb of the deleted region was inferred by comparison of two homologous genomic regions from poplar with a *Citrus* BES database. Structural data including gene content and order in the deletion was utilized for microsynteny and local gene colinearity studies concluding that in the studied region *Citrus* is more similar to *Populus* than to *Arabidopsis*, a phylogenetically closer species. This observation supports previous works on other species and suggests that *Arabidopsis* lineage underwent a quicker genome evolutionary dynamics than the *Populus* one.

ACKNOWLEDGEMENTS

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Mutagenesis as a Functional Genomics Platform for Pharmaceutical Alkaloid Biosynthetic Gene Discovery in Opium Poppy

I Desgagne–Penix, J M Hagel & P J Facchini*

Abstract

Opium poppy (*Papaver somniferum*) accumulates the analgesic benzylisoquinoline alkaloids morphine, codeine and thebaine, and remains one of the world's most important medicinal plants. The development of varieties that accumulate valuable compounds, such as thebaine and codeine, but not morphine precludes the illicit synthesis of heroin (*O,O*-diacetylmorphine) and has led to the establishment of alternative cash crops. Novel cDNAs encoding a growing number of biosynthetic enzymes have been isolated, and various –omics resources including EST databases and DNA microarray chips have been established. However, the full potential of functional genomics as a tool for gene discovery in opium poppy remains limited by the relative inefficiency of genetic transformation protocols, which also restricts the application of metabolic engineering for both experimental and commercial purposes. We are establishing an effective functional genomics initiative based on induced mutagenesis and recently developed reverse genetics methodology, such as TILLING (Targeting Induced Local Lesions IN Genomes), with the aim of identifying biosynthetic genes that can be used to engineer opium poppy for the production of copious levels of high-value pharmaceutical alkaloids. Mutagenesis involves the treatment of seeds with ethyl methane sulfonate (EMS) or by fast-neutron bombardment (FNB). In preliminary experiments with EMS-treated seeds, the screening of 1,250 independent M_2 plants led to the isolation of four mutants that displayed two distinctly altered alkaloid profiles. Two lines accumulated the central pathway intermediate reticuline and relatively low levels of morphine, codeine and thebaine compared to wild-type plants. Two other lines showed the unusual accumulation in the latex of the antimicrobial alkaloid sanguinarine, which is the product of a branch pathway distinct from that leading to morphine. The present status of –omics resources and functional genomics platforms available to study benzylisoquinoline alkaloid biosynthesis in opium poppy are discussed with a focus on the applications of induced mutagenesis.

Introduction

Opium poppy (*Papaver somniferum*) has long been one of the world's most important medicinal plants due to its unique ability to synthesize the analgesic narcotics morphine, codeine and thebaine in addition to a variety of other biologically active benzylisoquinoline alkaloids (BIAs) such as papaverine, noscapine, and sanguinarine. Alkaloids produced in opium poppy, like many specialized plant metabolites, are structurally complex and most cannot be economically synthesized. The licit market for morphine is in excess of one million kilograms annually, the majority of which is chemically converted to codeine. The lower potency of codeine relative to morphine makes it a more versatile drug found in several over-the-counter – in some countries including Canada, Australia and New Zealand – and prescription medications, such as pain relievers and cough syrup [1]. However, opium poppy produces relatively low quantities of codeine compared with morphine. The unfortunate side of this natural biochemistry is evident in the widespread and illegal cultivation of opium poppy for the production of heroin, which is derived via the relatively simple *O,O*-diacetylation of morphine. A thorough understanding of BIA biosynthesis in opium poppy provides an opportunity to customize the production of pharmaceuticals in metabolically engineered or mutagenized plants. Major targets are opium poppy varieties rich in thebaine, oripavine and codeine, but low in morphine. Thebaine and oripavine are feedstocks for the synthesis of valuable and powerful drugs, such as oxycodone (OxyContin), buprenorphine (Subutex), naloxone (Narcan) and naltrexone (ReVia), which are used as analgesics, and as a treatment for substance abuse and alcoholism. Paradigms established via an improvement of our knowledge of BIA biosynthetic pathways could also facilitate the metabolic engineering of other commercially or medicinally important plants.

Plant metabolic engineering typically involves the transfer of desired genes into the genome of a plant cell, followed by the regeneration of a transgenic plant from undifferentiated tissue. The genetic transformation of opium poppy has been reported, but remains a relatively difficult and inefficient procedure due primarily to recalcitrant regeneration [2–4]. With existing technology, opium poppy transformation protocols are applicable to research and development activities including the silencing and/or up-regulation of selected target genes, or the complementation of mutant varieties to confirm individual gene function. However, the establishment of a robust functional genomics platform and the rationale and comprehensive metabolic engineering of opium poppy will require a more reliable and efficient transformation system. An alternative to genetic transformation is the application of random and induced mutagenesis. A classic, *forward genetics* approach to acquiring plant lines with desired phenotypes involves the random mutagenesis of wild-type seeds using various chemical reagents or different forms of radiation. Such treatments generally introduce single nucleotide substitutions, whereas others cause deletions in the genome. Since some cells within each seed ultimately give rise to the next generation of seeds, the mutations in such cells are carried forward into future generations. The recent availability of myriad genomics resources for opium poppy facilitates the discovery of genes responsible for mutant phenotypes. Alternatively, mutations in genes of unknown function can be detected in plant genomes using recently established approaches, such as TILLING (Targeting Induced Local Lesions IN Genomes) [5]. The characterization of associated alterations in alkaloid phenotype facilitates the application of a potentially high-throughput, *reverse genetics* approach to gene discovery in opium poppy. In this paper, we discuss the prospects of using induced mutagenesis as a platform for both forward and reverse genetics in the context of BIA biosynthetic gene discovery in opium poppy.

Benzylisoquinoline alkaloid biosynthesis

BIA biosynthesis begins with the decarboxylation of tyrosine and dihydroxyphenylalanine (DOPA) by tyrosine decarboxylase (TYDC) to tyramine and dopamine, respectively (**Fig. 1**). TYDC constitutes

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a large gene family with about 15 members found in opium poppy [6]. Dopamine is the precursor for the isoquinoline moiety, whereas 4-hydroxyphenylacetaldehyde is incorporated as the benzyl component. The condensation of these tyrosine derivatives is a Pictet-Spengler type reaction catalyzed by the first committed step of the pathway, norcoclaurine synthase (NCS). The enzyme has been purified from *Thalictrum flavum* and corresponding cDNA have been isolated and functionally characterized from opium poppy and *T. flavum* [7–9]. NCS is related to the pathogenesis related protein (PR) 10 and Bet v 1 allergen protein families. However, homologous PR10 proteins from opium poppy are not catalytically active. Biochemical characterization of recombinant *T. flavum* NCS using a continuous enzyme assay based on circular dichroism spectroscopy following the generation of one enantiomer revealed a reaction mechanism involving a two-step cyclization with a direct electrophilic aromatic substitution [10].

Recently, a second enzyme producing the (*S*)-norcoclaurine enantiomer was isolated from *C. japonica* and displayed sequence similarity to 2-oxoglutarate-dependent dioxygenases [11]. It would be remarkable if two proteins belonging to different families were indeed involved in catalyzing the same reaction in the pathway.

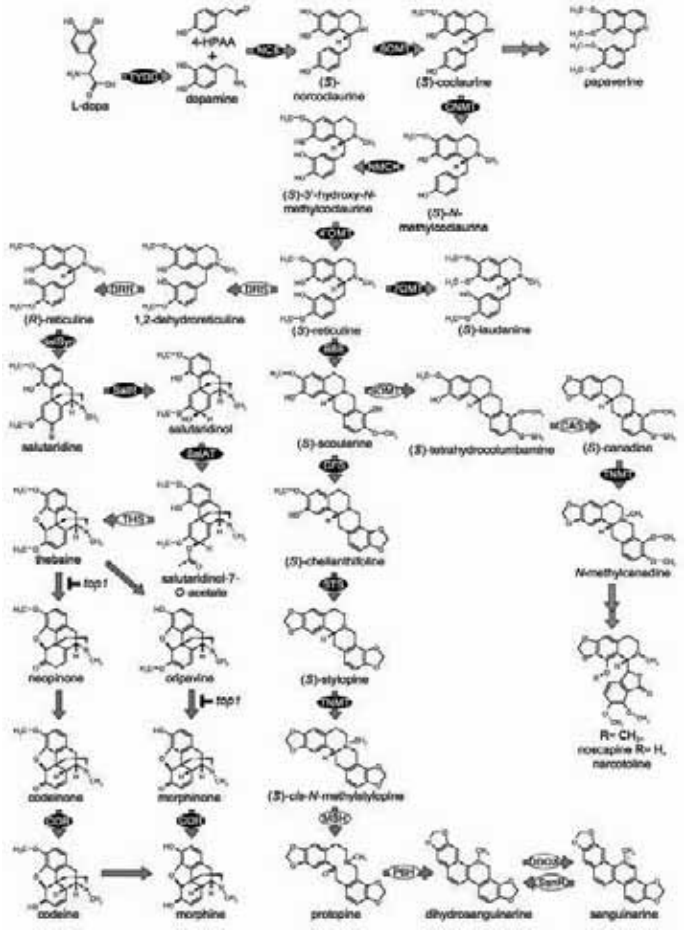

Figure 1 Benzylisoquinoline alkaloid biosynthesis in opium poppy. Enzymes for which cognate cDNAs have been isolated are highlighted in black, whereas white denotes those that have been characterized and/or purified. Abbreviations are provided in the text.

The conversion of (*S*)-norcoclaurine to (*S*)-reticuline involves *O*-methylation at position 6, *N*-methylation, 3'-hydroxylation, and a second 4'-*O*-methylation (**Fig. 1**). Norcoclaurine 6-*O*-methyltransferase (6OMT) and 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (4'OMT) are both class II *O*-methyltransferases that display strict regiospecificity. Cognate cDNAs have been obtained for each enzyme from opium poppy and *C. japonica* [12–14]. Coclaurine *N*-methyltransferase (CNMT) has been cloned from opium poppy and *C. japonica* and is more closely related to SAM-dependent cyclopropane fatty acid synthases compared with other *N*-methyltransferases [15,16]. The hydroxylation of *N*-methylcoclaurine is catalyzed by a P450 monooxygenase, *N*-methylcoclaurine 3'-hydroxylase (NMCH) [17,18].

(*S*)-Reticuline is the central pathway intermediate from which most BIA structural types are derived. Only the dimeric bisbenzylisoquinoline alkaloids are not produced via (*S*)-reticuline [19]. Another exception might be the biosynthesis of the simple *N*-demethylated BIA papaverine. The recent discovery of a 7-*O*-methyltransferase specific for norreticuline (N7OMT), but not *N*-methylated analogs, suggests that (*S*)-reticuline precursors might enter a pathway leading to the vasodilatator papaverine (J. Ziegler, personal communication). The papaverine pathway has not been empirically proven and represents a major target for alkaloid biosynthetic gene discovery in opium poppy. A second *O*-methyltransferase acting on reticuline and catalyzing the formation of laudanine was previously isolated from opium poppy [13]. (*R,S*)-Reticuline 7-*O*-methyltransferase (7OMT) does not accept *N*-demethylated BIA substrates, but is active towards phenolic compounds.

A major branch pathway that gives rise to many BIA classes begins with the formation of (*S*)-scoulerine by the berberine bridge enzyme (BBE) (**Fig. 1**). This enzyme has been cloned from several sources [20–22] and has recently been thoroughly characterized [23,24]. BBE belongs to a novel family of flavoproteins that possess two covalent attachment sites for FAD, one being histidine and the other cysteine. The cysteinylation of the cofactor was shown to increase the midpoint redox potential to a value higher than that observed for other flavoproteins thereby facilitating hydride abstraction of (*S*)-reticuline. This step represents the first half reaction toward the conversion to (*S*)-scoulerine. The biosynthesis of benzophenanthridine alkaloids is initiated by the formation of two methylenedioxy bridges resulting in (*S*)-cheilanthifoline via (*S*)-cheilanthifoline synthase (CFS) and (*S*)-stylopine through (*S*)-stylopine synthase (STS) (**Fig. 1**). Both reactions are catalyzed by P450-dependent monooxygenases and two cDNAs coding for stylopine synthase have been cloned from *E. californica* and classified as Cyp719A2 and Cyp719A3 [25]. Both recombinant proteins showed the same regiospecificity for methylenedioxy bridge formation, but Cyp719A2 only converts (*S*)-cheilanthifoline to (*S*)-stylopine. In contrast, Cyp719A3 also accepts compounds without a pre-existing methylenedioxy bridge. (*S*)-Stylopine is subsequently *N*-methylated to (*S*)-*cis*-*N*-methylstylopine by tetrahydroprotoberberine *N*-methyltransferase (TNMT). Based on homology to CNMT, a cDNA encoding TNMT was isolated and functionally characterized from opium poppy [26]. The enzyme shows a narrow substrate range in converting only tetrahydroprotoberberine alkaloids with dimethoxy or methylenedioxy functional groups at C2/3 and C9/10, respectively. TNMT is also one of only a few plant enzymes able to catalyze the formation of quaternary ammonium compounds. Subsequent hydroxylation by (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH) yields protopine, which is further hydroxylated by protopine 6–hydroxylase (P6H) to dihydrosanguinarine. Both enzymes have been detected in protopine alkaloid–containing cell cultures, and characterization suggests that they are P450 monooxygenases [27,28]. Dihydrobenzophenanthridine oxidase (DBOX), which converts dihydrosanguinarine to sanguinarine, was purified from *Sanguianaria canadensis* [29]. Sanguinarine reductase (SanR) catalyzes the reverse reaction [30].

The biosynthetic pathway leading to noscapine formation in opium poppy is unknown. However, this cough suppressant and potential anti–cancer drug could be derived via *O*-methylation of (*S*)-scoulerine by an enzyme related to scoulerine 9-*O*-methyltransferase (SOMT) (**Fig. 1**) [31]. A *C. japonica* cDNA encoding SOMT was shown to be involved in berberine biosynthesis [32]. Proposed noscapine biosynthesis would

require methylenedioxy bridge formation by a purported canadine synthase, an enzyme belonging to the Cyp719A family [25]. The remaining steps to noscapine formation are hypothetical [31] and represent novel targets for gene discovery.

Whereas all pathways downstream of reticuline begin with the (*S*)-epimer, conversion to the (*R*)-epimer of reticuline is a required entry step into the morphinan alkaloid biosynthetic pathway (**Fig. 1**). The epimerization of reticuline is a two-step process, involving oxidation of (*S*)-reticuline by 1,2-dehydroreticuline synthase (DRS) and subsequent formation to (*R*)-reticuline by 1,2-dehydroreticuline reductase (DRR). Both steps have been biochemically characterized and the enzymes partially purified [33,34]. Intramolecular carbon-carbon phenol coupling between C2 of the benzyl and C4α of the isochinoline moiety leads to the formation of salutaridine. The enzyme catalyzing this reaction, salutaridine synthase (SalSyn) was shown to belong to the P450 monooxygenase family [35]. SalSyn was recently cloned from opium poppy based on its higher expression in morphine containing *Papaver* species and functionally characterized (J. Ziegler, personal communication). The protein shows high homology to methylenedioxy bridge-forming P450-dependent enzymes, and was classified as Cyp719B1. The next step in the pathway is catalyzed by salutaridine reductase (SalR), and a cognate cDNA was obtained via the same approach used for SalSyn isolation [36]. Functional characterization of the enzyme showed the stereospecific reduction of the keto group to 7(*S*)-salutaridinol [37]. The enzyme belongs to the family of short chain dehydrogenases/ reductases (SDR) but, unlike many other enzymes in this family, exhibits a higher molecular weight and is monomeric. Stereospecific reduction of salutaridine is required for the next step, which is catalyzed by salutaridinol 7-*O*-acetyltransferase (SalAT). This enzyme specifically acetylates the 7(*S*)-epimer of salutaridinol to salutaridinol-7-*O*-acetate [38]. With considerable sequence homology to the acetylating enzymes from the MIA pathway, SalAT also belongs to the BAHD family of acetyltransferases [39]. The introduced acetyl group is eliminated, either spontaneously or by thebaine synthase (THS) [40], leading to the formation of an oxide bridge between C4 and C5 to yield thebaine, the first pentacyclic alkaloid of the pathway. The final steps in morphine biosynthesis consist of two demethylations and one reduction. Both the demethylations of thebaine to neopinone, which isomerises to codeinone, and codeine to morphine are not yet understood. Codeinone reductase (COR), which converts codeinone to codeine and morphinone to morphine, belongs to the aldo-keto reductase family and has been purified and cloned from opium poppy [41,42].

Genomics resources and functional platforms

Mutagenesis is a powerful approach for (1) the creation of new traits and (2) the identification of new genes. Populations of mutagenized plants provide a biological basis for the application of both forward and reverse genetics. In forward genetics, phenotypic screening is used to isolate mutants of interest and various tools and techniques are used to isolate the responsible gene. For example, a mutant population of opium poppy could be screened for qualitative or quantitative alterations in alkaloid profile. Mutagenized plant populations can also be subjected to reverse genetics, whereby plants with mutations in a specific target gene are identified followed by characterization of the associated phenotype.

Several methods are available for the creation of a mutagenized population. Plant transformation is required to establish a library of transfer-DNA (T-DNA) and/or transposon-tagged lines [5]. However, the limited efficiency of genetic transformation protocols for opium poppy effectively precludes the use of insertional mutagenesis. In contrast, procedures relying on the mutagenic properties of certain chemicals or ionizing radiation do not require transformation and can be effectively applied in most if not all plant species.

A generalized workflow involving forward genetics approaches for the purpose of gene discovery is illustrated in **Fig. 2**. Large populations of randomly mutagenized plants are first subjected to high-throughput phenotypic screening (i.e. metabolite profiling). Plants exhibiting phenotypes of interest are then subjected to further analysis aimed at identifying the mutated gene responsible for the observed traits. In plants where dense genetic maps or genomic sequence information are available, chromosome-walking techniques can be applied to narrow the search to a small region of the genome. Once the mutant phenotype has been linked with a relatively short genomic segment, BAC (bacterial artificial chromosome) or YAC (yeast artificial chromosome) clones harboring the DNA segment are used to narrow the search to a limited number of genes. Systematic expression of candidate genes in the mutant plant identifies the gene responsible based on the restoration of the wild-type phenotype.

In the case of opium poppy, mutants generated using chemicals or ionizing radiation can be screened for altered alkaloid profiles using high-throughput analytical methods (**Fig. 2**). However, genetic and physical maps are not yet available for opium poppy, precluding the use of chromosome-walking methodologies. Alternative measures, such as transcript profiling techniques (i.e. microarray analysis and comparative EST profiling) must be used to link a specific gene with an observed phenotype (**Fig. 2**). This strategy was used to isolate the *top1* mutant, (i.e. high levels of thebaine/oripavine and low levels of morphine/codeine) from a chemically mutagenized population [43]. Although it was proposed that *top1* harbors a mutation preventing the functional expression of an enzyme catalyzing the oxidative demethylation of thebaine and oripavine, comparative, microarray-based analysis of *top1* and its parent variety did not yield genes putatively involved in morphinan alkaloid biosynthesis.

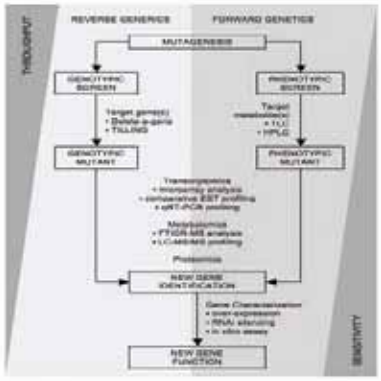

Figure 2 Generalized work-plan for forward and reverse genetics platforms to identify novel genes involved in benzylisoquinoline alkaloid biosynthesis using induced opium poppy mutants and various –omics resources.

Reverse genetics requires mutant populations for the purpose of isolating plants harboring specific genotypes rather than phenotypes. In model plants such as *Arabidopsis*, insertional mutants can be isolated using PCR-based methods. Briefly, primer sets designed against the T-DNA sequence and target gene regions are used to search for mutants with a target gene disrupted by a T-DNA insertion. In systems where insertional mutagenesis is not feasible, such as opium poppy, alternative approaches must be used for the genotypic screening of mutant populations, as illustrated in **Fig. 2**. Two screening methods are used, the choice of which depends on the mutagenesis procedure. Fast neutron bombardment (FNB) generally causes genomic deletions rather than nucleotide base transitions. To screen for deletion mutations in target genes, Delete-a-gene technology can be employed. This PCR-based approach involves screening pools of genomic DNA (gDNA) samples using primers flanking the target gene. The identification of deletions in specific

genes is improved by adjusting the PCR extension time to promote the preferential amplification of shorter amplicons [44,45].

In contrast, chemical mutagenesis with alkylating mutagens, such as ethylmethane sulfonate (EMS), causes point mutations. TILLING (Targeted Induced Local Lesions IN Genomes) is a high-throughput methodology used to screen pooled gDNA of chemically mutagenized plant populations to isolate nonsense or missense mutant alleles of target genes (**Fig. 2**) [46, 47]. Briefly, PCR is performed using fluorescently labelled and gene-specific primers, and the resulting amplicons are denatured and annealed. In gDNA pools where mutant alleles are represented, annealing of PCR products derived from wild-type and mutant alleles, respectively, leads to the formation of heteroduplexed DNA. Heteroduplexes are digested with the enzyme Cel-1, which is a nuclease specific for single-stranded DNA that recognizes mismatched base pairing. The fluorescent products are visualized after electrophoresis on denaturing polyacrylamide gels [5]. Generally, gDNA samples from M_2 plants are screened until an individual plant with a mutation in a target gene is identified. This plant and its progeny are then subjected to phenotypic analysis to determine gene function.

As illustrated in **Fig. 2**, genotypic mutants may be examined using targeted and broad-spectrum metabolite profiling based on, for example, liquid chromatography-tandem mass spectrometry (LC-MS/MS) or Fourier transform-ion cyclotron resonance mass spectrometry (FTICR-MS). Once a target gene has been identified using either forward or reverse genetics approaches, additional experiments must be performed to validate its function. For example, the gene can be heterologously expressed in bacterial or yeast systems and tested for enzymatic activity or other biological functions. Alternatively, post-transcriptional gene silencing (PTGS) techniques, such as RNA interference (RNAi) and virus-induced gene silencing (VIGS), can be used to characterize the function of candidate genes. Both RNAi and VIGS approaches have been used to investigate BIA biosynthesis in opium poppy. For example, RNAi was used to silence all members of the *COR* gene family (**Fig. 1**), which unexpectedly led to the accumulation of (*S*)-reticuline [48]. Similarly, RNAi suppression of *SalAT* resulted in the accumulation of salutaridine, the substrate of the enzyme immediately upstream of SalAT (**Fig. 1**) [49]. Both results suggest the occurrence of metabolic channels in BIA biosynthesis. Recently, the successful application of VIGS in opium poppy was reported [50], providing another tool for the characterization of gene function.

Integrated metabolomics

Underpinned by the tightly regulated expression of specific genes and the precise control of biosynthetic enzymes, plant metabolites are synthesized via an enormously complex biochemical network. Targeted phytochemical analysis has long been a basic component of plant metabolome research and modern approaches involving large-scale, broad-spectrum chemical profiling have facilitated the development of more complete and biologically meaningful metabolic models. As the final downstream product of the genome, the metabolome is defined as the total quantitative collection of low molecular weight compounds in a cell, tissue or organism [51]. The metabolome is chemically and physically more diverse than the transcriptome or proteome owing to large variations in molecular structures. Between 100,000 and 200,000 primary and secondary metabolites are estimated to occur in plants [52,53].

Metabolomics involves the use of high-throughput analytical strategies for the large-scale identification and quantification of metabolites [54]. A distinction is made between metabolomics and metabolite fingerprinting, which is used as a general sample classification tool and does not attempt to identify or quantify metabolites [55]. Both approaches require technologies capable of the rapid and comprehensive profiling of considerable numbers of compounds. Current analytical platforms include FTICR-MS and nuclear magnetic resonance (NMR) spectroscopy [56]. The detection of up to several thousand analytes (i.e. distinct masses) has been possible using FTICR-MS [57–59]. FTICR-MS remains a popular method for metabolomics due to its sensitivity and resolution, although the detected analytes usually outnumber the actual metabolites in the sample, and identification is highly equivocal. In contrast with the limited chemical information provided by FTICR-MS, ^{1}H NMR has the general capacity for unequivocal metabolite identification. Unlike MS-based approaches, ^{1}H NMR does not discriminate against certain chemical classes, and provides detailed chemical and structural information for each compound in a sample. However, since biological samples typically contain a large proportion of metabolites at low concentrations, the low sensitivity of ^{1}H NMR poses a drawback. While both FTICR-MS and ^{1}H NMR circumvent problems associated with sample fractionation prior to analysis, general limitations in instrument availability and high costs have contributed to the widespread use of separation-based methods, such as liquid/gas chromatography (LC/GC)-MS and capillary electrophoresis (CE)-MS. Until sensitive instrumentation with the capacity for high-throughput sample processing and compound identification is developed, comprehensive metabolome surveys require a combination of available technologies.

The ability to assemble 'snapshots' of biochemical processes by cataloguing large numbers of metabolites has become a valuable tool for plant functional genomics. In spite of the vast compilation of genomic data for numerous plant species, reliable gene annotation and functional assignment remain difficult [60]. The search for novel genes in non-model plants (i.e. those for which genome sequences are not available) has created expansive libraries of ESTs the predicted functions of which are based on homology to previously characterized gene products. The use of EST and proteomic resources in opium poppy has led to the identification, cloning and in vitro characterization of numerous enzymes [7,13,26,36]. For the purpose of gaining insight into the biological roles of unknown enzymes, metabolomics platforms can be employed in the analysis of plants exhibiting mutations in target genes (i.e. reverse genetics). Conversely, mutant chemical phenotypes identified via forward genetics approaches can be more thoroughly characterized using metabolomics-based methods. Beginning with the screening of large mutant populations, plants exhibiting potentially desirable chemotypes can be selected for more rigorous metabolite analysis (e.g. LC-MS/MS). Upon confirmation of the select phenotype (e.g. high codeine, high/low overall alkaloid levels) more detailed, broad-scope metabolite profiling can be performed, acquiring datasets, which can then be integrated with transcriptomic and/or proteomic methods to identify the genes whose mutation underlies the observed phenotype. A typical workflow involving the use of metabolomics for gene discovery in opium poppy is shown in **Fig. 3**.

Metabolomics has been used for mutant classification, the characterization of stress responses and plant-herbivore interactions, and the assignment of species and cultivars [61,62]. As an integral component of our functional genomics program, we have applied both FTICR-MS and ^{1}H NMR metabolomics to investigate metabolism in opium poppy. Alkaloid biosynthesis and accumulation are generally organ- and cell type-specific processes in plants. Sanguinarine typically accumulates in roots, whereas morphine, papaverine and noscapine are generally the most abundant alkaloids in aerial organs [63]. Most alkaloids accumulate in the multinucleate cytoplasm (i.e. latex) of articulated, secretory cells known as laticifers that form an internal secretory system under a positive turgor pressure similar to sieve elements [64,65]. Dedifferentiated opium poppy cell cultures do not constitutively accumulate alkaloids, but instead produce sanguinarine in response to treatment with a fungal elicitor [66]. Inducible sanguinarine production in opium poppy cell cultures is an excellent platform to characterize the activation of BIA pathways under controlled conditions. EST databases and DNA microarrays are among the genomics resources now available to discover new

alkaloid biosynthetic genes and relevant biological processes.

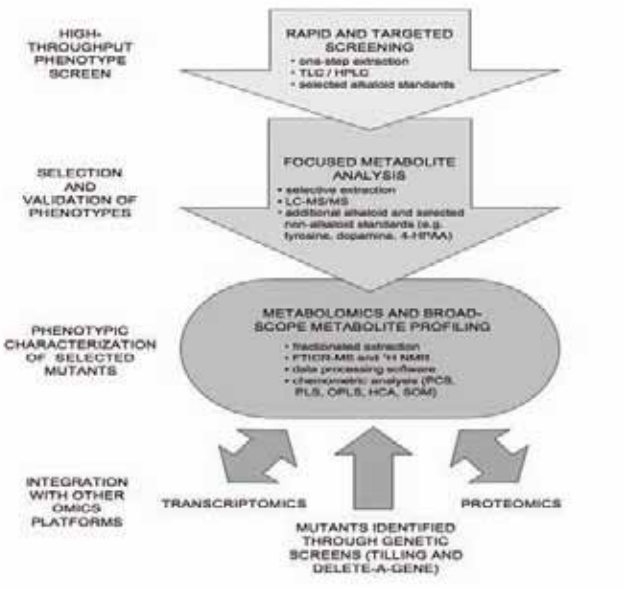

Figure 3 Workflow diagram showing methodologies and technical platforms used for different analytical approaches in metabolomics. Abbreviations and explanations are found in the text.

We have used FTICR-MS to show that substantial modulations in the metabolome of elicitor-treated opium poppy cell cultures are accompanied by major alterations in the transcriptome [59]. Several metabolites could be identified from 992 monoisotropic masses, including sanguinarine, dihydrosanguinarine, the methoxylated derivatives dihydrochelirubine and chelirubine, and the alkaloid pathway intermediates *N*-methylcoclaurine, *N*-methylstylopine, and protopine. The value of FTICR-MS for new compound discovery was demonstrated by the detection of low levels of chelirubine and papaverine, which were previously not reported in opium poppy cell cultures. Metabolite profiles of elicitor-treated cell cultures at most time points were distinctly separated by PCA, and some of the detected analytes exhibited temporal changes in abundance consistent with modulations in the profiles of alkaloid biosynthetic gene transcripts. The temporal shift in the accumulation of successive alkaloid intermediates in sanguinarine biosynthesis reflects biochemical flux through the pathway, with detectable intermediates representing potential regulatory bottlenecks.

In addition to FTICR-MS, ^{1}H NMR was used as a complementary approach to examine the metabolic response of opium poppy cell cultures to elicitor treatment [67]. Extensive reprogramming of primary metabolism along with an induction of alkaloid biosynthesis was seen using metabolite fingerprinting and compound-specific profiling. The levels of 42 diverse metabolites were monitored over a 100-hour period in control and elicitor-treated cultures. This approach permitted an impressive 70% success rate in the assignment of an absolute or relative quantity for 212 target compounds in the opium poppy cell culture metabolome. Combined with multivariate statistical analysis, targeted profiling revealed dynamic changes to the metabolome of elicitor-treated cells, especially in the cellular pools of sugars, organic acids and non-protein amino acids within 5 hours following elicitation. Substantial modulations were also observed in the metabolome of control cultures, particularly in the levels of amino acids and phospholipid pathway intermediates 80 hours after the start of the time course. Various flux modulations in primary metabolism were detected, such as glycolysis, the tricarboxylic acid cycle, nitrogen assimilation, phospholipid/fatty acid synthesis and the shikimate pathway, all of which provide precursors for specialized metabolism. Although both approaches were corroborative, more compounds were unambiguously identified using ^{1}H NMR compared with FTICR-MS.

Table 1. Examples of opium poppy 'mutants' with diverse alkaloid content

Phenotype	Variety	Reference
Alkaloid-free	Sujata	[75]
Low-morphine	Przemko	[72]
	Michalko	http://www.ihar.poznan.pl/mak2.htm
	Mieszko	http://www.ihar.poznan.pl/mak2.htm
High-thebaine	Norman (top1)	[43]
	Thebaine–rich	[73,74]
	SGE–29	[76]
	T	[68]
High-codeine	SG–35–I	[76]
	SG–35–II	[76]
	SGE–48	[76]
	SGE–9	[76]
High-morphine	Lazur	http://www.ihar.poznan.pl/mak2.htm
	Shweta	[75]
	Sanchita	[76]
	SN–2	[76]
	SE–1	[76]
	A1	[76]
	Kheops	[72]
High-noscapine	Kek Gemona	[72]
	Marianne	[71]
	Monaco	[72]
High-alkaloid	Tebona	[72]
	C048-6-14-64	[71]

NMR-based targeted profiling was also used to examine metabolism in latex and root tissues of mature opium poppy plants [68]. Previous examination of benzylisoquinoline alkaloid biosynthesis in poppy seedlings employed ESI-coupled MS_n and FTICR-MS techniques [69], and about 20 alkaloids were identified after feeding ^{13}C-labeled tyramine. In contrast, our ^{1}H NMR analysis was aimed at acquiring a more broad-scope perspective of the metabolome of natural poppy mutants, and was not restricted to alkaloids. Several varieties were screened to identify six candidates with unique alkaloid phenotypes. Proton NMR was performed on aqueous and chloroform extracts, and chemometric methods were used to compare the resulting spectra. Two mutants, including a low-alkaloid variety and a high-thebaine, low-morphine line, were clearly distinguished by multivariate analysis of the spectra acquired for latex extracts. We quantified 34 root and 21 latex metabolites and revealed significant differences in the accumulation of specific alkaloids in the latex of the low-alkaloid and high-thebaine, low-morphine varieties. Interestingly, few significant differences were observed in the levels of other metabolites, suggesting that the variation was restricted to alkaloid metabolism. This result was supported by quantitative real-time polymerase chain reaction (qRT-PCR) analysis of 42 genes involved in both primary and specialized metabolism, showing specifically that differential gene expression was largely associated with alkaloid biosynthesis. In exception to these trends, modulations in primary metabolism were evidenced by an accumulation of the alkaloid precursor tyramine in the low-alkaloid cultivar, along with reduced levels of sucrose, some

amino acids and malate. The metabolomics platform established by NMR for these natural mutants has allowed the rational design of comparative, DNA-microarray analysis of alkaloid biosynthesis in low-morphine (or low-alkaloid) plants versus high-morphine commercial cultivars. Lists of candidate genes putatively involved in morphine metabolism are now available for further study (J. Hagel and P. Facchini, unpublished data).

Mutants and mutagenesis

There are many different mutants of opium poppy, which are generally referred to as varieties or cultivars. Most have been selected through classical breeding on the basis of agronomically and ornamentally desirable traits (e.g. floral morphology and color, capsule size, resistance to environmental stress or pathogen challenge, seed oil content of seeds, and alkaloid content) (**Table 1**). For example the morphological mutant variety 'hens-and-chicks' displays a capsule phenotype whereby the base of one large central capsule is surrounded by a cluster of smaller capsules. Another example is the variety 'giganteum,' which produces large flowers and enormous capsules. In terms of alkaloid content, the cultivar 'Marianne' contains high levels of noscapine and narcotoline, in addition to morphinan alkaloids [68,70,71]. In contrast, cultivar 'Przemko' has a low overall alkaloid content [68,72] (**Table 1**). A natural mutant with reddish latex containing substantially higher levels of thebaine compared with morphine has been described [73,74] (**Table 1**). Natural and induced variations in opium poppy morphology and alkaloid profile almost certainly result from mutations in the genome.

Random genetic mutations occur spontaneously or can be induced using ionizing radiation or certain chemicals. Highly energetic particles (e.g. β-particles, neutrons and α-particles) or electromagnetic wavelengths can damage DNA. For example, fast neutron bombardment (FNB) induces mutation by deleting large regions of the genome leading to loss-of-function (i.e. knock-out) of genes located in those regions [44,45]. In contrast, chemical mutagenesis is much more localized and typically results in point mutations whereby one nucleotide is substituted with another. For example the chemical mutagen ethyl methanesulfonate (EMS) causes guanine alkylation leading to a transition-type mutation of the original G::C base pair, resulting in the formation of an A::T pair [77]. As such, chemical mutagenesis produces four types of mutation: (1) silent (i.e. occurring in an intron or non-coding sequence), (2) neutral (i.e. nucleotides change, but encode the same amino acid), (3) nonsense (e.g. introduction of a premature stop codon leading to a truncated protein), or (4) missense (i.e. a single nucleotide transition results in the substitution of a key amino acid). The latter two events can render the protein non-functional, or can alter the original function. Historically, opium poppy has been a popular target for mutagenesis using chemical alkylating agents and/or ionizing radiation [75,78–84]. For example, treatment of several generations of opium poppy seeds with gamma rays and EMS led to the isolation of mutants with variations in capsule dimension and morphine content [81,83]. More recently, EMS treatment produced the *top1* mutant, which accumulates thebaine and oripavine, but not morphine [43].

We treated 100,000 opium poppy seeds with 0.6% (v/v) EMS for 16 hours and promoted self-pollination to produce a M_1 seeds stock. The germination frequency in the field of the M_0 seed was approximately 15% resulting in the production of approximately 10,000 plants. From the seed obtained 10,000 M_1 plants were grown to maturity under self-pollinating conditions. A total of 1,250 dried and milled capsule samples were extracted using methanol and alkaloid profiles were determined by thin layer chromatography. Two alkaloid phenotype mutants were identified as shown in **Fig. 4**. One mutant (i.e. 2944) accumulated the central pathway intermediate reticuline and relatively low levels of morphine, codeine and thebaine compared with wild-type plants. Among other explanations, this alkaloid phenotype could result from modulation in the function of a putative transcriptional regulator controlling the expression of genes encoding biosynthetic enzymes acting on reticuline, the efficiency of a transporter responsible for the trafficking of reticuline to appropriate cellular compartments for further metabolism, or the catalytic activity of specific enzymes. Another mutant (i.e. 2650) showed the unusual accumulation in the latex of the antimicrobial alkaloid sanguinarine, which is the product of a branch pathway distinct from that leading to morphine. Available –omics resources provide opportunities to identify the genetic and biochemical basis for these interesting mutations in alkaloid phenotype.

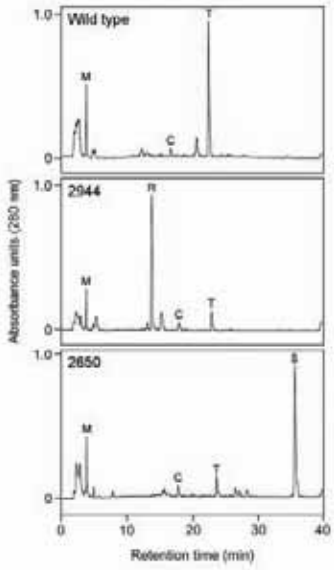

Figure 4 Mutants detected among 1,250 EMS-treated M_2 opium poppy plants. Abbreviations: C, codeiene; M, morphine; T, thebaine; R, reticuline, S, sanguinarine.

Future prospects

The ability to genetically engineer benzylisoquinoline alkaloid biosynthesis in opium poppy is highly desirable for myriad scientific, economic and social reasons. Plants produce a multitude of natural products, yet relatively little is known about the fundamental mechanisms that regulate the various biosynthetic pathways. In addition to various practical applications, the manipulation of specialized metabolic pathways via induced mutagenesis and genetic engineering offers a powerful tool to investigate the basic control mechanisms that regulate metabolic flux. The integration of the expanding repository of –omics resources with functional genomics strategies based on induced mutagenesis in opium poppy will substantially advance our knowledge of pharmaceutical alkaloid metabolism in this important medicinal plant. From an economic perspective, opium poppy, despite its notoriety, is a legal crop in many countries for the production of pharmaceutical morphine, codeine and other pharmaceuticals.

The development of technologies that emerge from this research could be licensed to opium poppy producers in other parts of the world. Moreover, opium poppy and related medicinal plant species could be developed into alternative crops in both developed and developing nations. Farmers in many countries need alternatives to commodity crops to remain competitive in an expanding global market. Genetic and metabolic engineering technologies offer immense potential to address these issues. Morphine produced from opium poppy is also the source of heroine, a narcotic drug with devastating social implications. Biotechnology must be considered an important weapon in the control of illicit drug trafficking. For example, drug cartels exploit poor farmers in developing countries for the production of the raw material from

opium poppy. One of the reasons that it is difficult to stem the supply of heroin coming out of Afghanistan, for example, is because the illicit cultivation of opium poppy offers farmers a higher financial return than the cultivation of commodity crops. Blocking alkaloid biosynthesis at codeine or thebaine could provide farmers around the world with a valuable crop with an alternative, licit market. However, successful implementation of any technological achievement requires political and socio-economic cooperation.

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From Discovery of High Lysine Barley Endosperm Mutants in the 1960-70s to new Holistic Spectral Models of the Phenome and of Pleiotropy in 2008

L Munck* & B Møller Jespersen

Abstract

As documented by eight IAEA/FAO symposia (1968-82) on nutritionally improved seeds, a wide range of high lysine endosperm mutants were isolated in maize, sorghum and barley. These mutants observed by new spectroscopic screening methods can now be exploited to advance basic biological research and theory. Since 1982, effective methods to overview the physiochemical composition of seeds by Near Infrared Spectroscopy evaluated by chemometric data analysis have developed. Spectroscopic analyses by calibration have now substituted the wet analyses in the industry. In genetics there has traditionally been a differentiation between major genes for qualitative and minor "polygenes" for quantitative traits. This view has been coupled with an incomplete understanding of pleiotropy. It is shown that seed spectra from isogenic barley endosperm mutants represent a coarse-grained physiochemical overview of the phenome that can be classified by chemometrics. Pleiotropy expressed by a gene is quantified as a whole pattern by the gene specific mutant spectrum subtracted by the spectrum of the parent variety. Selection for an improved plumpness (starch) in a breeding material with the *lys3.a* mutant visualizes in spectra the effect of enriching "minor polygenes" for an increased content of starch in a mutant gene background. Morphological, spectroscopic and chemical analyses suggest that mutant genes have both qualitative and quantitative expressions. They produce qualitative pleiotropic phenomenological patterns that can be observed as more or less severe changes in macro and microstructures of the plant and seed phenotype. Behind are quantitative chemical changes that by spectroscopy and chemometrics can be transferred to qualitative patterns. In fact, one major gene for a qualitative trait can act as several apparent minor polygenes for quantitative variables. This explains the reduced need for the previously expected several hundred thousands of genes and gene modifiers down to the about 30,000 genes that are now sequenced in barley.

Introduction

The use of endosperm mutants to analyze the total integrated effect of gene expression in seeds.

From 1968 to 1982, the IAEA/FAO staff in Vienna arranged eight symposia on seed protein improvement by mutation that highly stimulated research in this area during that time. In another article, we have reviewed the practical outcome in agriculture and industry of some important achievements in this research (see IAEA-CN-167-301). Here, we will focus on how the high lysine endosperm mutants in barley that were identified during that time could be exploited to evolve improved spectroscopic methods and genetic theory for understanding and controlling gene expression. This research is now again in an active stage that is likely to have a fundamental impact on genetics and molecular biology in the future. The development started when the senior author was involved in isolating the first high lysine barley mutant gene *lys1* in the Hiproly genotype at Svalöf, Sweden in 1967 [1,2]. As a screening method, a dye-binding (acilane orange) analysis for basic amino acids (lysine) was plotted against protein (N) in an x-y plot to select the outliers high in lysine. Doll and his group [3], working at the Risø National Laboratory, Denmark, then employed the dye-binding to protein (N) method to isolate a range of barley endosperm mutants in the 1970s including the very high lysine (+45%) mutant Risø 1508(lys3.a), as well as mutants with moderate to low increase in lysine such as the +20% mutant 8 (*lys4.d*) and Risø mutant 16, the alleles mutant 13 (*lys5.f*) and mutant 29 (*lys5.g*) that all are about +10% in lysine % 16/g N. The Risø mutant M-1508 (lys3.a) and the Hiproly lys1. genes were utilized at the Carlsberg Research Center, Copenhagen, Denmark in the 1970-90' s, in plant breeding [4] and for basic research. In Diter von Wettsteins group, the *lys3.a* was shown to be a regulatory gene [5] that inhibited synthesis of the hordeine proteins low in lysine by inducing a lack of demetylation of the promoter DNA for these genes. The electron micrographs [6] demonstrated in the *lys3.a* mutant a granular matrix of high lysine proteins that surrounded the endosperm protein bodies. They were drastically changed in size and microstructure (see **Fig. 3B**). The over-expression of water-soluble high lysine proteins in the two mutants was further investigated at Carlsberg in the group of the senior author in the 1970-90s by John Mundy, Robert Leah and Ib Jonassen [7]. Six high lysine seed proteins were identified. The first two, chymotrypsine inhibitor I and II, were over expressed in both *lys1* and in *lys3.a* phenotypes. The other four - a barley subtilisine alpha-amylase inhibitor, a protein synthesis inhibitor, a chitinase and a 1-3 ß-glucanase - were greatly increased in lys3.a. The six proteins were surprisingly identified as inhibitors of bacterial and fungal growth [7,8]. The barley mutant research was thus instrumental in opening up a new understanding of how the germinated barley seed in the field and in the malt house could obtain immunity towards microbial infections. Another line of biochemical research with barley endosperm mutants has focused on identifying structural genes for starch synthesis [9]. ADP-glucose is the substrate for starch synthesis in plants. It is synthesized outside the plastid by ADP-glucose phyrophosphorylases (AGPases). AGPase is a main regulator of starch synthesis. Risø mutant 16 lacks one of the AGPase isoenzymes, while the alleles *lys5.g* and *lys5.f* lack activity in one of the ADP-glucose transporters bound to the plastid [9].

In 1991, the senior author started a new research group on chemometrics [10,11]) and spectroscopy [11,12] at the Royal Agricultural University (now Life Science Faculty of University of Copenhagen). Here the barley endosperm mutant material from Risø, Carlsberg and Svalöf has been used as a test case for exploiting Near Infrared Reflectance (NIR) spectroscopy in plant breeding and in genetic research. The barley endosperm mutants evaluated by NIR spectroscopy can now for the first time be used to overview gene expression of specific endosperm genes in isogenic barley seed phenotypes with regard to physiochemical composition. The results facilitate a new complimentary view on the theory of gene expression in Systems Biology [14].

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Materials and Methods

The data set [13] consists of 92 endosperm mutant genotypes and normal barley controls grown in the field (n=23, **Fig. 1A, B, C, Fig. 2A**) and in the greenhouse (n=69, **Fig. 2B, Table 1**), and is described with spectroscopic and chemical analyses interpreted by chemometrics [10] and data inspection. The mutant Risø genotypes are the *lys3.a* (mutant 1508), *lys3.b* (mutant 18), *lys3.c* (mutant 19), *lys4.d* (mutant 8) mutant 16 and *lys5.f* (mutant 13) all in Bomi as well as *lys5.g* (mutant 29) in Carlsberg II. Mutants 95 and 449 in Perga are of Italian origin [13]. The *w1* (line 1201) and *w2* (line 841878) of unknown origin were imported to the Carlsberg collection assigned as *waxy* mutants [13]. Mutant *lys3.m* induced in Minerva originates from Carlsberg. Lysimax and Lysiba are starch and yield improved recombinants from crosses with *lys3.a* and normal barley from Carlsberg [4,12]. The chemical and Near Infrared Reflectance (NIR) spectral analysis (on milled flour 0.5mm sieve) was carried out by a Foss-NIRSystems (USA) 6500 instrument [12]. The raw spectra were Multiplicative Scatter Corrected (MSC) and presented as *log* 1/R absorption. Chemometric pattern recognition analysis was performed using Principal Component Analysis (PCA) for classification and Partial Least Squares Regression (PLSR) to chemical analyses for prediction [10].

Experimental: Recognizing sample and genotype specific NIRS patterns in barley seeds

The NIR spectra from the 23 barley seed sample in **Fig. 1A** depict 1400 wavelength variables with a seemingly narrow variation between samples in absorption value (MSC *log*1/R). Classical statistics of variance based on distributional assumptions cannot extract information from such a complex whole inter-correlated data matrix with thousands of wavelength variables per sample. For this purpose, there is a need for self-modeling multivariate chemometric data models such as PCA for classification and PLSR for prediction based on latent variables. We found that the PCA in **Fig. 1B** calculated from the spectra in **Fig. 1A** differentiated between structural and regulative mutants and classified the spectra into three distinct populations: normal (N) barley, regulative endosperm (P) mutants with a high lysine percentage in protein and structural carbohydrate (C) mutants with low starch content. The C barley lines such as Risø mutant 16, *lys5.g* and *lys5.f*, that are structural starch mutations of DNA for the AGP-ase starch synthesis and transport mechanisms, have been used by the biochemists to study starch synthesis. It was therefore surprising when we found that these mutants and three others - mutant 95, mutant 449 and the *w1* (line 1201) were all placed in the C cluster (**Fig. 1B**) and that the loss of starch was largely compensated by an overproduction of ß-glucan (BG). *w1* has earlier been wrongly classified as a *waxy* mutant but is a ß–glucan compensating low starch mutant. *w2* is here classified, as normal barley, but is *waxy* and is high in amylopectin. It can be concluded that *lys4.d* is likely to be a regulative P mutant because it is classified together with the regulative mutant *lys3.a* [5] and its alleles *lys3.b*, *lys3.c* and *lys3.m* (**Fig. 1B**). The mean BG value for the C (12,3%), P (3,7%) and N (4.7%) classes are marked in **Fig. 1B**. Obviously, there is a strong pleiotropic regulative effect where mutations in starch metabolism may channel glucose from α- to ß-glucan production [13].

PCA classifications, that are useful for an overview of large (spectral) data sets, are most often published in literature without inspecting the underlying data structure because the significance of individual spectra tends to be underrated in mathematical modeling. We generated the mean spectra for the C, P and N genotypes and visually screened the whole spectra. Marked differences were found in the narrow area 2260-2360 nm (**Fig. 1C** marked "a" in **Fig. 1A**) of chemical and genetic significance. The near infrared reflectance spectra (NIRS) represent in principle a physical-chemical fingerprint containing repetitive information on the propensity of chemical bonds [12]. A trained spectroscopist can from the first, second derivatives or by MSC of the *log*1/R NIRS data directly explore specific chemical differences between samples and deduce destructive chemical analyses for verification. The P and C genotypes have a characteristic bulb in the area from 2336 to 2352 nm which in spectroscopic literature is assigned to cellulose (2336 and 2352nm) and fat (2347nm). The substantial increases of the fat components anticipated from the mean spectral patterns of C and P barleys in **Fig. 1C** are verified by chemical analyses in **Table 1**. The reproducibility, fine-tuning and informative capacity of NIRS spectra are indeed impressive. The MSC *log*1/R absorption range is 0.04 units for classification of C, P and N barleys in **Fig. 1B**. However, the range needed is 100 times less for classifying the C versus P+N groups in the 1890-1920nm area for dry matter content within the narrow response of 89-93% d.m. (Munck, 2007 p.412, 414, [14]). A high BG content of the C group conditions a mean difference in dry matter of 1.5% between these groups.

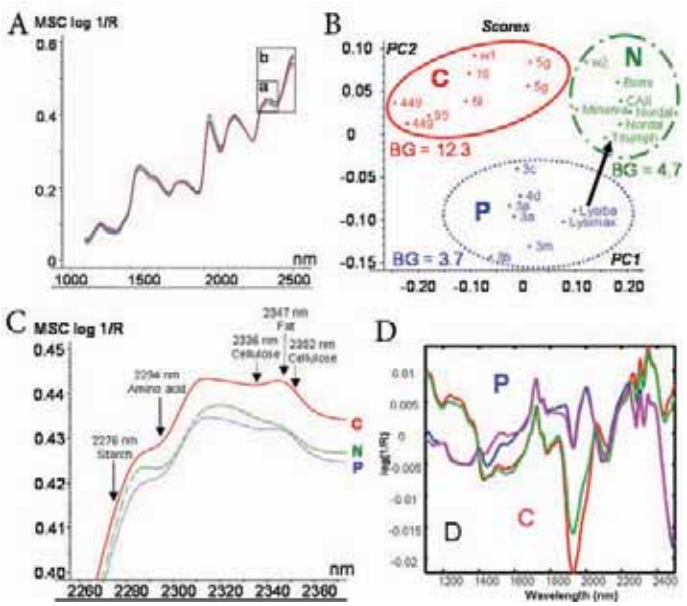

Figure 1 Classification of mutant material (n=23) by Principal component analysis(PCA) of Near Infrared Reflectance(NIR) spectra, and by chemical analysis (Munck 2005 [11]). **(A)** Barley seed NIR spectra 400-2500. **(B)** A PCA scoreplot of an endosperm mutant material. Note pos. of *lys3.a* versus *lys3.a* recombinants Lysimax/Lysiba. **(C)** Mean spectra 2260-2360nm for normal(N) barley and protein(P), carbohydrate mutants(C) in B. **(D)** Representation of pleiotropy; mean centered differential MSC *log*1/R spectra 1100-2500nm to parent line Bomi for the P mutants *lys3.a* (blue), *lys4.d* (magnenta) compared to the C mutants *lys5.f* (green) and Risø mutant 16 (red).

The precision of NIRS allows visualization of individual barley mutant gene expression by the differential, subtracted spectral patterns to their parent variety. This is demonstrated by the four differrential spectra MSC *log*1/R 1100-2500nm in **Fig. 1D** with the chemical evaluation shown in **Table 1**. They represent the P mutant's lys3.a, and *lys4.d* as well as the C mutant *lys5.f* and Risø mutant 16, all in Bomi grown in greenhouse. It is clearly seen that the two pairs of C and P mutants are differentiated and finely tuned. The seemingly small differences between the members of each pair is highly reproducible when the material is grown in a controlled environment and can be further analyzed by chemometrics and spectral evaluation to be interpreted by chemical analyses (**Table 1**) for verification. The question is now if the NIR selection screening method can be used in plant breeding also involving minor genes? In **Fig. 1B** and in IAEA-CN-167-301 it is demonstrated that the spectral tool can be used in practical "data breeding" from a PCA score plot to select for improved recombinants. The positions of the original mutant *lys3.a* in **Fig. 1B** are moved to the right for Lysimax and Lysiba demonstrating an improved background to the *lys3.a* gene for seed plumpness and for improved starch [4]. The differential spectra 2260 to 2460nm (area a.

Fig. 1A) to Bomi of the improved Lysiba and Lysimax genotypes and of the original *lys3.a* mutant and its *lys3.c* allele in Bomi are presented in **Fig. 2A**. The spectral differences between the alleles *lys3.a* and *lys3.c* at 2460nm are mainly due to ß-glucan (3.1 and 6.4% respectively). The mean starch content of the two *lys3.a* recombinants Lysimax and Lysiba was improved by 4.9 absolute percent in relation to the *lys3.a* genotype when grown in the same field. The spectra from the two starch-improved *lys3.a* lines are moved downwards to the baseline and the characteristic mutant pattern is flattened out approaching Bomi representing a straight line representing 16 years of plant breeding at Carlsberg 1976-90.

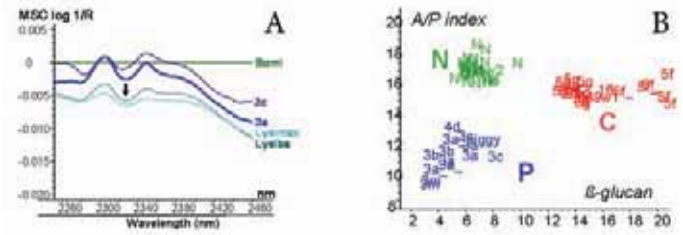

Figure 2 (A) Differential spectra 2260-2480nm to Bomi for the isogenic *lys3.a* and *lys3.c* mutants compared to the starch improved *lys3.a* recombinants Lysimax and Lysiba. **(B)** Chemical classification of the 69 barley lines by ß-glucan versus the Amid/protein A/P index [11].

It was also surprising to us that it was possible to collapse the 1400 NIR spectral points into three chemical analyses ß-glucan (x) and the amide/protein A/P index (y) in a x-y plot to obtain just as good a separation of the N, C and P barley genotype classes in **Fig. 2B** [11] as in the PCA score plot in **Fig. 1B**. While protein alone has a low power in discriminating between the genotypes, the amide-to-protein (A/P) index clearly separates the high-lysine P mutant *lys3.a* from the others. Ratios seem more indicative for genotype classification than pure variables. This was first demonstrated by the dye-binding (DBC) to Protein (N) plot [1,11] that was used to select the first high lysine mutant Hiproly at Svalöf and that was employed in large scale at Risø [3] to find a collection of mutants. We will hereafter discuss how the new insight in pleiotropy by NIR spectroscopy explains why it is possible to detect mutants with a very complex gene expression by just simple linear plots and ratios [11,12,14].

The barley endosperm mutant NIR-spectral seed model as a tool for a reformed integrated view on the pleiotropic expression of qualitative and quantitative genes.

In genetics there has traditionally been a differentiation between major genes for qualitative and minor "polygenes" for quantitative traits, coupled to an incomplete understanding of pleiotropy. Now our results from NIR spectroscopy of seeds from barley endosperm mutants makes it clear that all active genes are both contributing to and dependent on the internal "genotypic milieu," as already forecasted by Chetverikov in 1926 [15]. The qualitative morphological seed trait of the *lys3.a* mutant can be described as shrunken seeds with an enlarged scutellar plate (K) in **Fig. 3A**, and a drastically changed microstructure of the protein bodies of the endosperm in **Fig. 3B** [6]. Odd Arne Olsen and his group in the 1980-90' s at the Agricultural University, Ås, Norway, pioneered research in understanding the pleiotropic histological effects in the seed tissues [16] of the different mutants selected as high lysine by the dye-binding to protein (N) method. Both DNA-regulative P protein mutants such as *lys3.a* and DNA-structural C starch mutants such as Risø mutant 16 showed pleiotropic effects involving the diploid (embryo/scutellum) and triploid (aleuron and starchy endosperm) tissues of the seed. In scutellum, both mutant types displayed larger cells than Bomi and had starch granules in the embryo that were absent in the control. In the endosperm, all high lysine mutants except lys1. and mutant 7 had smaller cells. An influence of giberellic acid stimulation was found to be pleiotropic to the *lys3.a* gene, that was suggested to be in a state of "premature" germination. The Risø mutant 16 and the lys3.a genes are indeed "major" genes that involve cell structure and have drastic effects on the chemical composition (**Table 1**). However, also not so drastic "minor" biochemically specific mutants seem to have a secondary effect on the microstructure of tissues. Thus, the proanthocyanidine "biochemical" mutants of barley developed at Carlsberg in the 1980-90' s by Diter von Wettstein and Barbro Strid are devoid of the testa layer from the inner seed coating [5]. In order to relate the morphological qualitative traits of the Risø mutant 1508 in **Fig. 3A and B** to quantitative chemical traits (**Table 1**) by spectroscopy, the gene specific MSC *log*1/R NIR spectra 1700-1800nm for seeds grown in the field for the *lys3.a* mutant and its parent variety are displayed in **Fig. 3C**. The two spectra of the *lys3.a* mutant demonstrate a high reproducibility. They deviate in spectral pattern from that of Bomi. The peaks at 1724 and 1762 nm for *lys3.a* are assigned by experiments in the spectroscopic literature to represent unsaturated fat that is increased from 1.7% in Bomi to 3.5% in *lys3.a* (**Table 1**). To demonstrate that NIR spectra represent patterns of chemical variables or bonds, the NIR wavelengths in the interval MSC *log*1/R 1680-1810nm of the barley material (n=92) [11,12,13,14] were correlated to starch, ß-glucan, protein and amid nitrogen. The correlation coefficients are presented in **Fig. 3D** to explain the chemical significance of the mutant spectra in **Fig. 3C**. The chemical bonds assigned to specific wavelengths found in literature are presented below in **Fig. 3D**. It is verified that the chemical correlation spectra obtained by simple correlations in **Fig. 3D** are finely differentiated and tuned with highly significant positive and negative correlation peaks. Dividing the material in two separate datasets confirmed a high level of reproducibility and fine-tuning. It is characteristic that chemical spectra built on multivariate iPLS correlation coefficients, computed on the same data material with intervals as short as 5nm, is too destructive and cannot obtain a fine tuning comparable to the single wavelength correlation coefficients of the chemical spectra in **Fig. 3D**. Gentle data treatment to reduce physical scattering in spectra (e.g. MSC), followed by spectral inspection in the tradition of the classical spectroscopist Karl Norris [12,14], is therefore absolutely necessary to avoid losing information. However, data compression by chemometrics is essential for classification (**Fig. 1B**) and to isolate the "hot spots" in data.

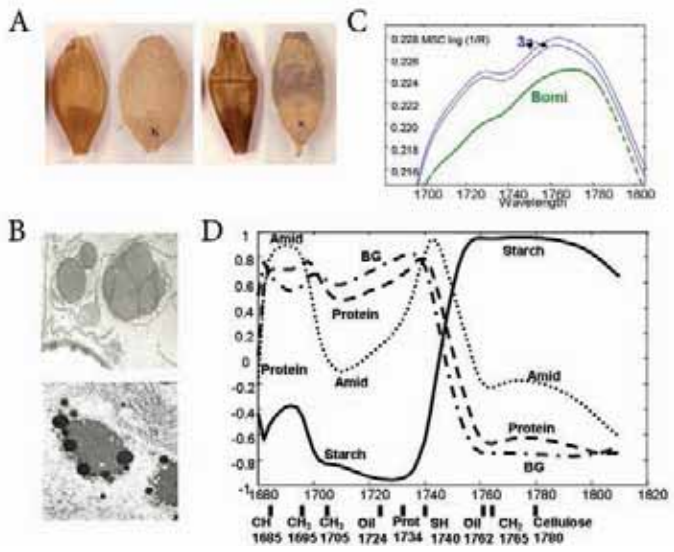

Figure 3 The morphological, microstructural,spectroscopic and chemical interpretation of the *lys3.a* and Bomi phenotypes. **(A)** Covered and dehulled kernels of Bomi/left and lys3.a/right. K= scutellar plate. **(B)** Electron micrographs of Bomi/above and lys3.a/below endosperm protein bodies (Munck and v.Wettstein 1974). **(C)** MSC *log*1/R NIR spectra 1700-1800nm of Bomi and lys3.a. **(D)** Chemical Interpretation of the NIR interval 1680-1820nm by simple correlations for each wavelength.

Fig. 3D represents the richness in chemical information in just a small region of the NIR spectrum. Now, for the first time, NIR spectral representations for the pleiotropy of a mutant gene and for the seed phenome can visualize the concerted action of all genes expressed in "the genotypic milieu" as pleiotropy in Chetverikovs sense. Bossinger, et al [17], in their paper on the genetics of plant development at the IGBS-VI symposium in Helsingborg, Sweden in 1991, analyzed the classical Svalöf mutant material developed by Åke Gustafsson and Udda Lundqvist. By different morphological classes of mutants, they demonstrated that phytomeric morphological units stepwise regulate plant development. We may here look upon the endosperm tissue in each seed as a self-organizing cellular computer [11,14] that is integrating and adding to the information brought to the seed in the form of assimilate. It reflects the specific phytomeric development from seedling stage to plant maturity. The stunning reproducibility of the spectra from identical genotypes grown under the same conditions [12,14] shows that gene pleiotropy for both plant and seed development mutants can be studied with respect to their effect on seed composition in an isogenic background by NIR spectroscopy.

Table 1. Chemical composition in Greenhouse for BG, Starch (S) BG+S, Protein, Amid, Fat, Lysine and Glutamic acid (Glu)

	n	Betaglucan	Starch	BG+S	Protein	Amide	Fat	Lysine	Glu
Bomi	2	7.0±0.3	47.0±2.6	54.0±2.3	15.7±1.6	0.38	1.7[a]	3.27[a]	22.9[a]
lys3a	2	4.5±1.2	40.7±1.3	45.2±0.1	17.70.9[b]	0.320.03[b]	3.5[a]	4.94[a]	14.86[a]
lys4d	1	4.0	41.1	45.1	17.5	0.37	-	4.04	19.46
lys5f	2	19.8±0.2[b]	30.0±0.78	49.9±0.9	17.0±1.4[b]	0.42±0.06[b]	3.7[a]	3.32[a]	27.59[a]
mutant 16	2	15.2±0	29.9[a]	46.1[a]	17.1±1.5	0.45±0.06	-	3.37[a]	22.5[a]
lys5g	5	13.2±0.7	39.0±8.1[c]	52.4±8.2[c]	17.8±1.2	0.44±0.04	2.1[a]	3.60±0.23[c]	21.56±1.10[c]

[a]n=1, [b]n=3, [c]n=2

The classical genetic notion of considering separate minor polygenes and "heritability" for quantitative traits cut out from the biological network by destructive chemical analyses produces a need for a great number of genes with specific chemical expression. The combined microscopic and spectroscopic analysis in **Fig. 3** suggests that all specific genes and gene mutations have both qualitative and quantitative effects. They produce qualitative pleiotropic phenomenological patterns that can be observed as more or less severe changes in the macro and microstructure of the plant and seed phenotypes. Behind are quantitative chemical changes that by spectroscopy and chemometrics can be transferred back to visualize the hidden gene specific, qualitative chemical patterns that earlier have not been considered in genetics. They can now be compared to the morphological ones and interpreted by molecular methods for classification. In fact, one major gene for a qualitative trait is able to act as several apparent minor polygenes for different quantitative variables. This explains the reduced need from the previously expected several hundred of thousands of specific genes and gene modifiers down to the order of about 30,000 genes that are now sequenced in barley.

In conclusion, in the future, micro-spectroscopy and spectral imagining analysis combined with the new directed mutant techniques for TILLING (Targeted Induced Local Lesions IN Genomes), also presented in this volume, will represent a new promising combination of analytical hardware. This model, applied in a controlled gene background, represents new possibilities to explore and with a great reproducibility, to define a pleiotropic spectral qualitative gene expression for each mutant gene on the level of the phenotype (spectral phenome). The coarsely grained spectral information will be chemically and molecularly quantified and validated by new integrated computerized methods for spectral pretreatment and spectral inspection and with improved data modeling software.

ACKNOWLEDGEMENTS

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The Effect of Plants with Novel Traits (PNT) Regulation on Mutation Breeding in Canada

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Abstract

The Canadian Environmental Protection Act (1988) has within it a definition for biotechnology. This definition would have allowed the government department, Environment Canada, to regulate all genetically modified organisms (GMOs) in Canada. In response to this, the Canadian Food Inspection Agency (CFIA), which reports to the Minister of Agriculture and Agri-Food Canada, developed the concept of a Plant with Novel Trait (PNT). Not only does this definition capture GMOs, it also includes induced mutations, natural mutations and exotic germplasm that have not previously been grown in Canada. It is a system that is product, not process based. However, apart from questions regarding the novelty of traits in new plant varieties, breeders are asked by CFIA to identify the process used to develop the trait or traits in question. Field trials involving breeding lines with a PNT may be subject to confined testing. This conference celebrated 80 years of unconfined development and testing of induced plant mutations. This regulation is time consuming, expensive and an innovation barrier for Canadian plant breeding. It can only be hoped that other nations, and particularly those that have successfully used induced mutations, will not emulate Canada's approach.

Introduction

The announcement for this meeting summarized the use of mutation techniques in plants over the past 80 years and the positive economic impact this has had throughout the world. During the meeting we have learned of the direct release of varieties of crop plants from mutation breeding and varieties developed with genes identified in mutant crop plant populations. We have also heard about the use of site directed mutagenesis, which would avoid the regulatory hurdles associated in most countries with genetically modified organisms (GMOs). We were told that TILLING (Targeted Induced Local Lesions IN Genomics) would also not be caught in the regulatory trap. However, this use of mutations does not avoid regulation in Canada. How Canada came to be in this position and the regulation's effect on plant breeding is the subject of this presentation.

CEPA

In 1988 the Parliament of Canada passed the Canadian Environmental Protection Act (CEPA) into law. Within this Act is a definition for biotechnology, which reads: *the application of science and engineering in the direct or indirect use of living organisms or parts or products of living organisms in their natural or modified forms*. The definition was placed in CEPA to deal with concerns regarding genetically modified organisms (GMOs) and allow Environment Canada to regulate all GMOs as well as varieties arising from traditional plant breeding techniques. It is interesting that biotechnology is the only technology defined in CEPA, and this reveals the shaky ground on which the definition was built and its spurious danger to the environment.

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CFIA response to CEPA

Government departments do not like to have responsibilities taken from them and, in this case, the danger that it poses is towards production agriculture in Canada. From 1988 and onwards, Environment Canada had the potential to regulate the registration of crop varieties in Canada, a responsibility that was then held by a branch of Agriculture Canada. In anticipation of the regulatory issues CEPA would cause, Agriculture Canada and the Ministry of State for Science and Technology sponsored a Workshop on the Regulation of Agricultural Technology organized by the Canadian Agriculture Research Council (CARC). CARC was a council of agriculture researchers from industry, universities and federal and provincial governments and they invited a number of respected Canadian scientists to the workshop. The workshop developed 18 recommendations that were sent to various government ministries including Agriculture Canada. The two key recommendations that applied to crops were:

- *The products of biotechnology should be regulated, not the process that produces them and*
- *The definition of biotechnology in use by the regulatory agencies is too broad and must be redefined with more focus. The plant sector, for example, would limit it to genetic engineering.*

By 1997 the responsibility for registering crop varieties had passed on to a reorganized group of Agriculture Canada known as the Canadian Food Inspection Agency (CFIA). The CFIA has organized a number of workshops over the years to explain the regulation of GMO's in Canada, one of which took place at the University of Saskatchewan in 2004. An official of the CFIA in his presentation stated:

1. *The CARC workshop arrived at several key recommendations*
2. *The product, not the process should be regulated. Those plants which possess characteristics or traits sufficiently different from the same or similar species should require an assessment of risk. (http://www.inspection.gc.ca/english/plaveg/bio/consult/novnou/watsone.shtml)*

However, as regards point 2, the CARC meeting actually recommended:

Products Subject to Regulation:

"Genetically modified organisms (GMO), which possess characteristics or traits that are 'sufficiently different' from the characteristics or traits of previous members of the same or similar species so as to require a separate assessment of risk".

Definitions for the above

Genetically modified organisms: organisms which are obtained by in vitro alteration of genetic material including, but not restricted to, recombinant DNA, nuclear and organelle transplantation, or genetic manipulation of viruses.

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Despite the best advice of CARC and members of the Canadian Plant breeding community, in 1994 Agriculture Canada issued regulatory directive 94-08, which contained the following concept and definition:

Plant with Novel Trait: A plant variety possessing a characteristic that is intentionally selected or created through a specific genetic change and is either not previously associated with a distinct and stable population of the cultivated plant species in Canada or expressed outside the normal range of a similar existing characteristic in the plant species.

So what exactly is a plant with a novel trait (PNT) in Canada? CFIA defines it as:

A PNT is a plant that contains a trait that is both new to the Canadian environment and has the potential to affect the specific use and safety of the plant with respect to the environment and human health. These traits can be introduced using biotechnology, mutagenesis, or conventional breeding techniques and have some potential to impact weediness, gene flow, plant pest potential, non-target organisms, or biodiversity. (http://www.inspection.gc.ca/english/plaveg/bio/pbobbve.shtml)

Canada now has a system that not only regulates GMOs but also is able to regulate traditional breeding techniques as well as induced mutation! This, despite there being no evidence that induced plant mutations have caused harm to humans, animals or the environment. We are told that Canada's variety registration system is science based but there was no review of the science of mutation breeding in the development of the PNT definition. Despite assurances from CFIA and the recommendation of CARC, we do not have a product based regulatory system. We now have a system in which both product (novelty) and process (GMO) is regulated. In the Procedures for the Registration of Crop Varieties in Canada published by the CFIA the pedigree of the proposed variety is required such that:

In the case of varieties resulting from recombinant gene technology, information on the gene(s) inserted, its source and gene products must be provided. Exact DNA sequence information must be provided to facilitate the generation of gene probes for variety verification purposes. http://www.inspection.gc.ca/english/plaveg/variet/proced/regproe.shtml

In the Variety Registration Application Form, the developer is specifically asked: *does this variety contain traits that are novel to its species?,* is referred back to Regulatory Directive 94-08.

Examples of the effect of PNT regulation

The "Flor de Mayo" bean is a particular market class of *Phaseolus vulgaris* L. that has a pink, marbled seed coat color and is not unlike the pinto or cranberry classes grown in Canada. It is a popular market class in Mexico, which has export potential for Canada. However, there is no variety of "Flor de Mayo" bean registered for production in Canada. The Crop Development Center (CDC) at the University of Saskatchewan developed a variety of "Flor de Mayo" and applied for a Canadian registration of the variety in April of 2002. After considering the application, the CFIA asked the CDC to prove that it wasn't a PNT since this "type" had never been grown in Canada. Since the market potential for Saskatchewan grown "Flor de Mayo" beans was judged to be too small, the CDC determined that the costs associated with demonstrating the "safety" of this market class were not worth it. Consequently, the application to register the variety was withdrawn.

The experience of the CDC in the registration of the low phytate barley, HB379, is an example of other difficulties that the PNT definition has produced. The low phytate character is one that was often referred to at this conference, as it is an example of mutant genes being used for improving the nutritional and environmental quality of some crop plants. The CDC had developed a low phytate barley, HB379, and applied to CFIA for registration in May 2006. There was no indication from CFIA that this was a PNT but there was a concern that it was a "novel feed." The concept of a novel feed was one that arose out of the novel plant concept and is administered by the CFIA. It took 17 months and hundreds of man-hours before HB379 was finally registered for production in Canada.

Conclusion

The concept of PNTs as developed and applied in Canada is time-consuming, expensive and an innovation barrier for Canadian plant breeding. It is a threat to the constructive use of plant mutations for crop improvement and it is to be hoped that other countries will not follow Canada's example.

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Turning Plant Mutation Breeding Into a New Era: Molecular Mutation Breeding

Q Y Shu

Abstract

Advances in molecular genetics and DNA technologies have brought plant breeding, including mutation breeding, into a molecular era. With ever-increasing knowledge of molecular genetics and genomics and rapidly emerging molecular techniques, breeders can now use mutation techniques in breeding new varieties more wisely and efficiently than ever before. Plant molecular mutation breeding is here defined as mutation breeding, in which molecular or genomic information and tools are used in the development of breeding strategies, screening, selection and verification of induced mutants, and in the utilization of mutated genes in the breeding process. It is built upon the science of DNA damage, repair and mutagenesis, plant molecular genetics and genomics of important agronomic traits as well as induced mutations. Mutagenic treatment, super-mutable genetic lines, molecular markers and high throughput DNA technologies for mutation screening such as TILLING (Targeting Induced Limited Lesions IN Genomes), are the key techniques and resources in molecular mutation breeding. Molecular mutation breeding will significantly increase both the efficiency and efficacy of mutation techniques in crop breeding. A perspective molecular mutation breeding scheme is proposed for discussion.

Introduction

Plant breeding is often regarded as applied genetics, and so is mutation breeding. One of the most important breakthroughs in the history of genetics was the discovery of experimental mutagenesis in the early 20th century, which later brought about plant mutation breeding. Without knowing much of the molecular biological basis, a vast amount of genetic variability was induced in the most economically important plant species, and a small portion of those variations induced has resulted in the development of more than 3,000 mutant varieties worldwide in about 180 plant species during the past 60 years [1]. With the unprecedented development of plant molecular genetics and functional genomics during the past decade, scientific exploration of induced mutation in plants has progressed dramatically from basic research on mutagenesis in plants, to the development of advanced genomics-based technologies, to their unique applications in gene discovery and development of novel crop traits [2]. These developments are bringing plant mutation breeding into a new paradigm – Plant Molecular Mutation Breeding.

Genetics and features of classical mutation breeding

In principle, plant breeding methods can be classified into three systems: recombination breeding, mutation breeding and transgenic breeding, each with unique way of generating variation and of selecting target lines (**Table 1**). In terms of mutation breeding, the generation of new mutated alleles is the core and most unique feature. The genetics behind mutation breeding includes differences in the sensitivity of different genotypes and plant tissues to different mutagens, often measured using lethal doses (LD), genetic chimeras after mutagenic treatment and their effect on transmission of mutated alleles and segregation in the following generation, and also the often recessive nature of induced mutations. Such knowledge of genetics is important for establishing proper doses and modes of mutagenic treatment, as well as for the methodology of harvesting and growing M_2 populations (**Table 1**).

Mutation breeding has its advantages and limitations. The advantages include creation of new gene alleles that do not exist in germplasm pools, and the induction of new gene alleles for a commercial variety so new varieties carrying desired mutation alleles can be directly used as a commercial variety. The limited genetic changes of any single plant of a mutated population and the often recessive nature, enable breeders to develop a new variety in a short breeding cycle. The disadvantage of mutation breeding is its limited power in generating the dominant alleles that might be desired; it is also less effective than cross breeding for a trait needs for a combination of multiple alleles, such as tolerance to abiotic stresses. The low mutation frequency requires growing and screening a large population for selection of desired mutants at a reasonable confidence. This becomes very expensive for traits that have to be evaluated through laborious phenotypic analysis.

Table 1. Genetics of three unique breeding methods for seed crops

	Recombinant breeding	Mutation breeding	Transgenic breeding
Source of genetic variation	Recombination of gene alleles from parental varieties.	New alleles artificially and randomly created from endogenous genes.	Insertion of new genes or modification of endogenous genes.
Transmission, expression and inheritance	No selective transmission; co-segregation of closely linked alleles.	Induced mutations subject to diplontic and haplontic selection.	Expression of transgenes subject to position effect or silencing.
Nature of gene action	Dominant, recessive alleles, and QTLs.	Mostly recessive alleles.	Mostly dominant alleles.
Breeding generations*	About 10 generations.	About 2-3 generations.	About 3 generations.

* the number of generations needed to obtain a homozygous line

Molecular genetics and genomics related to mutation breeding

The rapid development of plant molecular genetics and genomics in areas relevant to mutation breeding has been reinvigorating this breeding method; it is expected that mutation breeding will directly benefit from the rapid scientific and technological advances in molecular genetics and genomics.

DNA damage and repair

It has been well documented that DNA is subject to continuous damage and the cell has an arsenal of ways of responding to such injury; although mutations or deficiencies in repair can have catastrophic consequences

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for organisms, mutations are nonetheless fundamental to life and evolution [3]. With the accumulating knowledge of the molecular genetics of DNA damage and repair, we can now elucidate many of the phenomena that we have observed in classical mutagenesis, e.g. the differences of sensitivity to different mutagens among plant species and among plant materials. There are different pathways for the repair of DNA damages caused by different types of mutagen, for example, gamma irradiation often leads to DNA double strand breaks (DSBs, [4]), ultraviolet (UV) radiation results in covalent dimerization of adjacent pyrimidines [3], while chemical mutagens cause miss-pairing or nucleotide excision. This knowledge is very important for properly designing mutagenic experiments in a way that an enhanced mutation frequency can be achieved. For example, there are two pathways in DSB repair: homologous recombination (HR) and non-homologous end-joining (NHEJ; also known as illegitimate recombination). HR repair is quite precise and results in few mutations, while NHEJ is an error–prone process and thus can produce mutations [4]. Therefore, a genetic line defective in HR repair, or haploid materials such as pollen or anther (lack of homozygous DNA template for HR) is expected to produce a high frequency of mutations after radiation treatment. Such knowledge may also provide clues to identify new chemicals that can induce mutations in plants while having limited toxicity to humans.

Molecular genetics of induced mutations

Cells with damaged DNA will survive only when these damages are repaired either correctly or erroneously; the result of erroneous repairs will be fixed in the genome as induced mutations. The nature of DNA damage caused by different types of mutagens to a great extent determines the molecular feature of induced mutations. For example, the chemical mutagen EMS often leads to mutations of G/C to A/T transition [5], while ion beam implantation could cause deletion of DNA fragments of various sizes [6]. Although information is so far limited in this field, such knowledge will definitely help choose the proper mutagen for different purposes in mutation breeding. For example, DNA deletions in most cases will cause recessive mutations, while nucleotide substitution may produce a dominant allele. Therefore, when a recessive mutation could solve the problem, irradiation might be a better choice, while when a dominant mutation is needed (for example for herbicide resistance), a chemical mutagen might be more useful. It is also important for establishing proper methods of DNA-based mutation screening.

Molecular genetics of target trait

In general, plant molecular breeding depends on the understanding of the molecular genetic control of target traits of interest. Molecular genetic information is also of great help in developing a proper mutation breeding strategy. First, it is important for assessing the feasibility and potential to induce a mutation of interest. Since the mutation frequency for any given fragment of DNA or gene is more or less similar, the opportunity to obtain a mutant of different traits would therefore be dependent on the number of genes that control the trait. For example, many genes can affect the growth duration (i.e. days from sowing to heading for cereals), therefore the mutation frequency of such trait is often far higher than single-gene controlled traits [7]. Second, a mutation may have pleiotropic effects if the gene is at the upstream or at the middle of a long biosynthetic pathway, such as the MIPS gene in phytic acid biosynthesis and precautions should be taken for such a mutation project. Third, knowledge of genes controlling a trait of interest would constitute the very basis of the TILLING (Targeting Induced Limited Lesions IN Genomes) method.

Perspective of molecular mutation breeding

With more knowledge of DNA damage, repair and mutagenesis becoming available, more traits of interest being dissected at the molecular genetics level, and more molecular techniques developed and commanded by breeders, mutation breeding will be transformed into a new paradigm. A perspective scheme is proposed with the potential to significantly enhance the efficiency of plant mutation breeding (**Fig. 1**).

Effective mutation induction

Mutation induction is the starting step in mutation breeding, and its low frequency has been a severe limiting factor. Equipped with knowledge of the DNA damage and repair, we should be able to design strategies of mutagenic treatment to significantly increase the mutation frequency. For example, smartly combined use of chemical and physical mutagens, and recurrent mutagenic treatments, would increase mutation frequency, since theoretically each time of treatment would cause DNA damage which should be repaired after treatment, and consequently introduce new mutations each time. We should be able to select a suitable mutagen and starting material for a specific purpose, for example, chemical mutagens should be more suitable for inducing dominant alleles while physical mutagens might be better used for recessive mutations.

When the genes responsible for correct DNA damage repair are knocked out or mutated, these lines could become highly sensitive to mutagenic traetment and mutation frequency could be significantly increased. Such lines are commonly called "super mutable genetic lines", they could be generated through genetic transformation, i.e. silencing the genes using RNAi technology (**Fig. 1**). Once mutants of important traits are induced and identified using such super mutable lines, the transgenes and mutated genes can be separated by self-crossing since they are not linked to each other, hence non-transgenic stable mutant lines could be produced (**Fig. 1**). This has already been proven in *Arabidopsis* by silencing the mismatch repair gene AtMSH2 [8]. Similarly, suppression of the HR system through a transgenic or classic mutation approach is expected to enhance the NHEJ repair, and hence induce mutations at a higher frequency when treated with physical mutagens that could cause DSBs.

We should also be able to produce super mutable plant lines by genetic modification of specific genes in DNA repair system as shown [8]. Similarly, suppression of the HR system could enhance the NHEJ repair and hence induce a higher mutation frequency when treated with physical radiations.

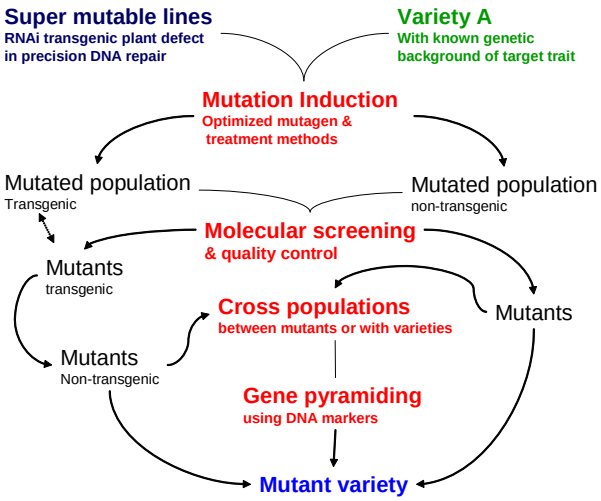

Figure 1 Plant molecular mutation breeding scheme.

High throughput mutation screening

Mutation screening has been another bottleneck in mutation breeding, particularly for traits that cannot be visually identified and have to be

assessed by costly or laborious chemical tests. This is being changed due to the establishment of DNA-based mutation screening techniques during the past few years [2].

The TILLING system [9], for example, based on the CEL I cleavage of mismatches, has been successfully exploited in several plant species [2]. A variety of modified versions of TILLING have already become available, such as that for detection of deletions --de-TILLING [10]. It is foreseen that mutation screening technology will become more high throughput, powerful and affordable with the rapid development of DNA technologies including high throughput DNA sequencing techniques.

Mutation utilization

In classical mutation breeding, induced mutations are embedded in mutants that are either directly or indirectly (through crosses with other varieties) used for developing a new variety, whereby it is rather difficult to trace the mutated genes in subsequent breeding. It is now possible to tag mutated genes, pyramid them into a single elite breeding line, and follow them up in subsequent breeding programs (**Fig. 1**).

When the genes responsible for correct DNA damage repair are knocked out or mutated, these lines could become highly sensitive to mutagenic treatment and mutation frequency could be significantly increased. Such lines are commonly called "super mutable genetic lines", they could be generated through genetic transformation, i.e. silencing the genes using RNAi technology (Fig. 1). Once mutants of important traits are induced and identified using such super mutable lines, the transgenes and mutated genes can be separated by self-crossing since they are not linked to each other, hence non-transgenic stable mutant lines could be produced (Fig. 1). This has already been proven in *Arabidopsis* by silencing the mismatch repair gene AtMSH2 [8]. Similarly, suppression of the HR system through a transgenic or classic mutation approach is expected to enhance the NHEJ repair, and hence induce mutations at a higher frequency when treated with physical mutagens that could cause DSBs.

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Molecular Genotyping of GA3 Insensitive Reduced Height Mutant of Emmer Wheat (*Triticum dicoccum*)

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Abstract

Emmer wheat (*Triticum dicoccum* Schubler) is cultivated in parts of peninsular India. Grains of emmer wheat contain higher amounts of protein and dietary fiber and hence are recommended for inclusion in the diet. Traditional varieties of emmer are tall, susceptible to lodging and low yielding. An induced semi-dwarf mutant was obtained in tall emmer wheat variety NP200. The seeds of variety NP200 were subjected to 100, 200, 300 or 400Gy of γ-rays. In the M_2 population of 200Gy treatment, a reduced height mutant with vigorous growth and high tillering was observed. The reduced height mutant, its parent and other emmer varieties were tested for their response to GA_3 treatment in seedling test. The mutant was found to be insensitive to externally applied GA_3. The mutant, its parent and tall and semi-dwarf varieties of emmer were subjected to *Rht* genotyping. Allele specific primers for dwarfing gene (*RhtB1b*) and their wild type allele (*RhtB1a*) were used. The validity of primers in emmer varieties was confirmed. All semi-dwarf emmer varieties showed a band of 237bp with primer pair BF-MR1. The mutant (HW1095) showed absence of amplification for both *RhtB1a* and *RhtB1b* alleles with respective primer pairs, indicating that the mutant carried a different mutation than the existing allele (*RhtB1b*). The mutant allele was amplified with another primer pair resulting in a product of about 400bp. In a comparative yield trial, the mutant gave higher yield than the other emmer wheat.

Introduction

Emmer wheat (*T. dicoccum*) is reported to contain high amounts of protein and dietary fiber [1,2] and hence is recommended for inclusion in diet [3]. Emmer possesses better resistance to wheat rust and is more tolerant to high temperature than other species of wheat [4,5]. Traditional varieties of emmer are tall, susceptible to lodging and low yielding [6]. Tall emmer variety NP200 is a selection from local wheat. Introduction of semi-dwarf stature has resulted in improvement in harvest index and yield in bread wheat. To date, 21 height reduction genes are known in wheat, including two major genes *RhtB1b* and *RhtD1b* which are present in 90% of the semi-dwarf cultivars [7]. Search for alternative height reducing genes led to the discovery of genes *RhtB1d* and *Rht8* (located on 2DS) from varieties Saitama-27 and Akakomughi respectively. Introduction of dwarfing gene *RhtB1b* has also shown improvement in emmer wheat [8]. It seems possible to generate variability for reduced height genes to increase options for breeders to improve emmer wheat. In this study, investigations on a γ-ray induced short stature mutant HW1095 are reported.

Materials and Methods

Seeds of emmer wheat variety NP200 were subjected to 100, 200, 300 and 400Gy of γ-rays. Treated seeds were space planted in the M_1 generation, allowed to self-pollinate and were harvested individually. In the M_2 population of 200Gy treatment, a reduced height mutant with vigorous growth and high tillering was observed, and carried forward as plant to row progeny. Subsequently, its progeny was designated HW1095. The mutant was tested in yield trials in five states representing peninsular and central parts of India.

GA_3 insensitivity test

Twenty seeds were placed in blotting paper folds supported by stands. Seedlings were grown either in water (control) or 10^{-4}M GA_3, their height was recorded after 12 days, and differences in seedling height of GA_3 treated and control plants were analyzed using Student's t-test.

Rht genotyping

DNA was isolated from bulk of five plants according to [9]. The mutant along with parent (NP200), tall variety (NP201) and semi-dwarf emmer varieties (DDK1009, DDK1025, HW5013, HW5301 and MACS2961) were subjected to *Rht* genotyping. The alleles *RhtB1b* and *RhtB1a* were amplified using allele specific primer combinations: primer BF: (5'-GGTAGGGAGGCGAGAGGCGAG-3') and MR1: (5'-CATCCCCATGGCCATCTCGAGCTA-3') for *RhtB1b*; primers BF and WR1 (5'-CATCCCCATGGCCATCTCGAGCTG-3') for *RhtB1a* [10]. DNA from the mutant along with tall and semi-dwarf emmer varieties was also amplified with a different primer pair, which differs from perfect pair at forward end (5'-TCTCCTCCCTCCCCACCCCAAC-3'). The PCR reaction was performed in 25µl containing 10pmoles of each primer, 100µM of dNTPs, and 2mM of $MgCl_2$, 1 unit of *Taq* DNA polymerase and 100ng of template DNA in an Eppendorf Mastercycler. The PCR products were separated on a 2% agarose gel prepared in 1X TBE buffer, visualized under UV light after Ethidium bromide staining and photographed [11].

Results and Discussion

The mean height of parent was 110cm, the mutant was about 35% shorter at mean of 71cm. There was no major segregation in the mutant for height in subsequent generations. In general, the mutant resembled the parent in morphology and was about three days early in flowering. In addition, the mutant showed reduced lodging.

The reduced height mutant along with its parent and other emmer varieties were tested for their response to externally applied GA_3. Height of control (water grown) seedlings of parent was 25.4±0.6cm while GA_3 treated seedlings were 35.9±1.4cm. There was 29.2% ($p \leq 0.01$) increase, which was significant ($p \leq 0.01$). Difference in seedling height of control (water grown) (18.80.4cm) and GA_3 treated (19.2±0.5cm) for the mutant was not significant. This showed that the mutant was insensitive to GA_3 treatment. The mutant seedlings were 45.6% lower in height as compared to the parent after GA_3 treatment. Another tall emmer variety NP201, showed 24.2±0.3 and 30.4±0.6cm for control and treatment, respectively. The difference (20.4%) ($p \leq 0.01$) was significant. The corresponding values for the semi-dwarf emmer varieties DDK1009, DDK1025,

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HW5013, HW5301 and MACS2961 were 16.8±0.2, 18.7±0.5, 17.1±0.3, 16.6±0.3 and 16.3±0.4 for control, and 16.0±0.2, 18.4±0.4, 16.1±0.4, 14.5±0.3 and 15.2±0.5 for GA_3 treatment, showing no significant difference ($p \leq 0.01$).

Emmer is gaining importance and yield improvement is needed. Reduction in plant height has been found to be effective in improving yield in high input conditions in durum and bread wheat. Transfer or induction of dwarfing genes has been useful in imparting lodging resistance and resulted in improvement of harvest index. Semi-dwarf varieties of emmer wheat have been developed, however, there is scope to induce mutation for this trait.

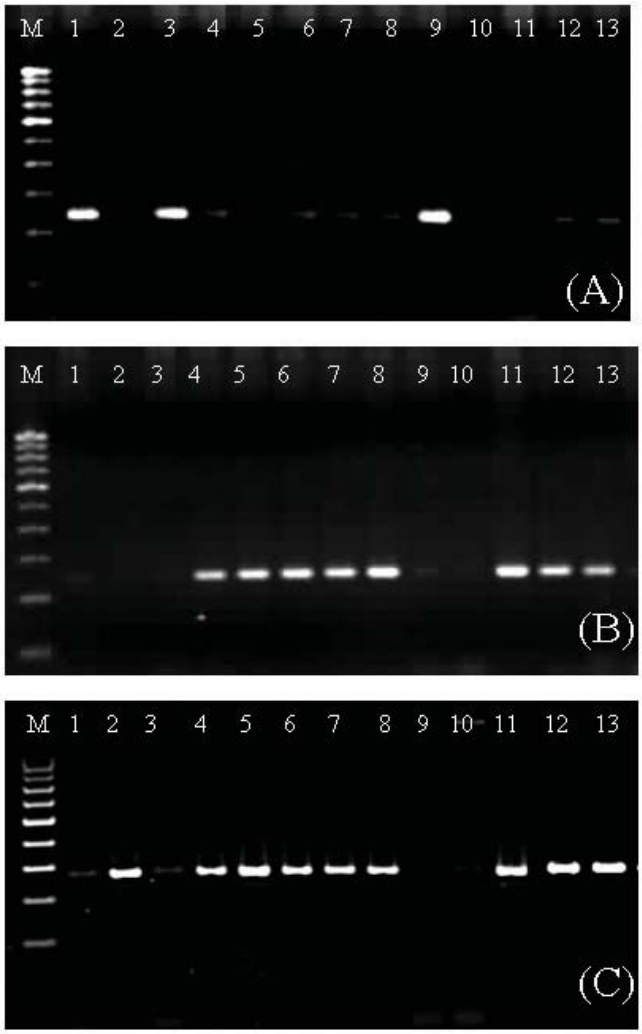

Figure 1 PCR analysis of *T.dicoccum* varieties and *T.dicoccum* (mutant) and *T. durum* varieties. Lane M: 100 bp ladder; Lane 1 & 3: *T.dicoccum* tall varieties-NP200 & NP201; Lane 2: *T.dicoccum* mutant HW1095; Lanes 4-8: *T.dicoccum* dwarf varieties - DDK1009, DDK1025, HW5013, HW5301, MACS2961; Lanes 9 & 10: *T.durum* tall varieties- A-9-30-1 & Bijaga Yellow; Lanes 11-13: *T.durum* dwarf varieties-HD4502, HD4530 and MACS2846: with primer BF-WR1 for RhtB1a **(A)**, BF-MR1 for RhtB1b **(B)** and new primer pair (5'-CTCCTCCCTCCCCACCCCAAC-3') and (5'-CATCCCCATGGCCATCTCGAGCTA-3') **(C)**.

A reduced height phenotype in the material under investigation can result from introgression of the known gene *RhtB1b* or from an induced mutation. To check these possibilities, allele specific markers for *RhtB1b* developed according to [10] were used. The semi-dwarf durums (HD4502, HD4530, and MACS2846) showed amplification of *RhtB1b* (Fig.1, B). Allele specific markers were also used to check the status in tall emmer (NP200 and NP201), five semi-dwarf emmer varieties (DDK1009, DDK1025, HW5013, HW5301 and MACS2961) and the newly developed semi-dwarf mutant (HW1095) (Fig.1, A & B). Use of allele specific markers has not been reported earlier in emmer wheat. The validity of primers in tall emmer varieties for *RhtB1a* and semi-dwarf emmer varieties for *RhtB1b* was confirmed. All semi-dwarf emmer varieties showed a band of 237bp with primer pair BF-MR1. The parent variety NP200 showed amplification of wild-type allele (*RhtB1a*), however, the mutant (HW1095) showed absence of amplification for both *RhtB1a* and *RhtB1b* alleles with respective primer pairs. This indicated that the mutant carried a different mutation than the existing allele (*RhtB1b*). This was further confirmed by amplifying with another primer pair which covers a longer stretch of DNA segment (**Fig. 1C**). In the mutant, this primer amplified a product of about 400bp as compared to 237bp with perfect primer pair (BF-MR1). These results are consistent with another study where this primer was reported to amplify the mutant allele which was not amplified by perfect primer pair [13]. The amplification will enable further characterization of induced mutation. A variation was also observed in tall durum cultivar Bijaga Yellow, where a band of 237bp was expected with the primer pair BF-WR1, however, no amplification was observed with either primer pairs (**Fig. 1, A & B**).

A comparative yield trial, which included eight released varieties of emmer, one bread wheat and one durum as check varieties, NP200 parent and the mutant HW1095, was conducted in five diverse locations [12]. The results showed that the mutant gave highest yield in three of the five locations among all the varieties. The mutant gave higher yield than the parent and other emmer wheat varieties at all the locations (**Table 1**).

Table 1. Grain yield of reduced height mutant (HW1095), parent and released varieties across different emmer growing regions of India

Genotype	Gujarat State (qt/ha)	Karnataka State (qt/ha)	Maharashtra State (qt/ha)	Tamil Nadu State (qt/ha)	Peninsular Zone (qt/ha)	Mean of All Zones (qt/ha)
DDK1028	22.7	37.8	37.9	22.1	37.6	33.2
DDK1030	23.5	39.7	41.2	0 7. 2	40.3	33.6
DDK1009	23.5	38.5	40.2	29.4	39.2	35.1
MACS-2947	26.8	39.5	40.9	27.2	40.0	36.2
DDK1025	21.7	42.5	39.4	31.8	44.1	36.3
MACS-2956	25.4	40.0	39.6	35.9	39.7	36.6
DDK1029	31.5	41.4	46.5	10.9	43.5	37.9
MACS-2961	26.8	42.7	46.0	23.4	44.1	38.6
NP200 (parent)	22.6	34.4	42.7	20.6	37.9	33.2
HW1095 (mutant)	29.0	43.3	49.1	21.6	45.8	40.0
MACS-2846 (*T.durum*)	34.7	38.6	46.4	12.4	41.8	37.5
MACS-2496 (*T.aestivum*)	45.3	40.0	47.0	12.5	43.2	40.4
S.E. mean	0.64	0.66	1.08	0.44	0.44	
CD	1.8	1.8	3.0	1.3	1.2	

The study reported here showed that an induced mutation resulted in reduction in height and insensitivity to externally applied gibberellin. The absence of amplification with primers specific to wild-type allele or the known dwarfing genes and GA_3 insensitivity indicated that there was a different mutation at the *RhtB1* locus.

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Genetic Analysis and Gene Mapping of Dwarf Mutant Rice CHA-1

H Wang*, Z Chen, T Guo, Y Liu & H Li

Abstract

The dwarf mutant rice CHA-1 which is studied here, is a stable and inherited rice material selected from the induced generation derived from rice variety Tehuazhan, which had been flown on a high space balloon. In order to investigate the inheritance of the dwarf genes in CHA-1, crosses between CHA-1 and tall variety Huiyangzhenzhuzao were carried out. Genetic analysis of the height in the F_2 generation showed that the new mutant gene (temporarily named *h*) from CHA-1, was a major recessive gene which linked with *sd-1* to a certain extent. The two genes had complementary interaction and together controlled the dwarf trait of CHA-1, which therefore was *hhsd-1sd-1*. To map the locus of the dwarf gene *h*, bulked-segregate analysis and recessive-class analysis in the F_2 generation from the cross between CHA-1 and variety 02428 were used to screen SSR molecular markers linked with the dwarf gene. Results showed that the dwarf gene *h* was located on chromosome 1, with a genetic distance of 4.715 cM from SSR marker RM302 and a genetic distance of 5.915 cM from the semi-dwarf gene *sd-1*.

Introduction

Plant height character is one of the most important agronomic traits of rice. Far-ranging utilization of the semi-dwarf gene *sd-1*[1] had made a great breakthrough in the rice production. This great achievement is well known as the "green revolution" in the breeding history of rice. However, the frequent use of a single gene source was hiding inherited brittleness. Thus, identification and utilization of non-*sd-l* gene sources is an important subject for present rice breeding. Space mutation is one of the effective ways to create new dwarf rice germplasms. The special dwarf mutant rice CHA-1 was obtained by space mutation. Compared with its original variety, the main agronomic characters of CHA-1 showed strong variation. The plant height decreased to only half of the original, 55~65centimeters. At the same time, positive variations were found in terms of panicle number, grain length-width rate, inartificial outcrossing rate and exterior quality. In addition, the hybrids which cross between CHA-1 and different tall varieties showed significant heterosis. In order to identify and apply the new dwarf rice source CHA-1 in rice breeding, genetic analysis and mapping of the dwarf gene *h* were studied.

Materials and Methods

Materials

- Dwarf mutant rice CHA-1 (*indica*)
- Tehuazhan (original variety, had semi-dwarf gene *sd-1, indica*)
- Aijiaonante (had semi-dwarf gene *sd-1, indica*)
- Huiyangzhenzhuzao (high variety, *indica*)
- 02428 (had semi-dwarf gene *sd-1, japonica*)

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Methods

7022 resin slides of the neck internodes from adult plants of CHA-1, Tehuazhan and Aijiaonante were made, and the longitudinal structures of them were examined under the microscope (40×).

A cross between CHA-1 and Huiyangzhenzhuzao was carried out, and then the height of every adult plant of the F_1 and F_2 generations measured.

Bulked-segregate analysis and recessive-class analysis in F_2 generation from the cross between CHA-1 and variety 02428 were used to screen SSR molecular primers which are distributed throughout rice chromosomes, and find the SSR markers linked with the dwarf gene *h*. Finally, the genetic map of the dwarf gene *h* was constructed.

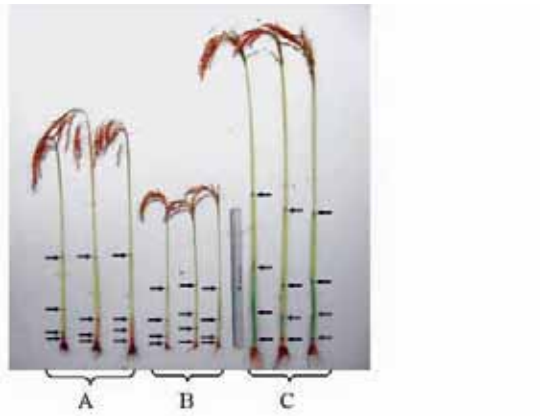

Figure 1 Elongation pattern of internodes. **(A)** Aijiaonante, **(B)** CHA-1, **(C)** Tehuazhan.

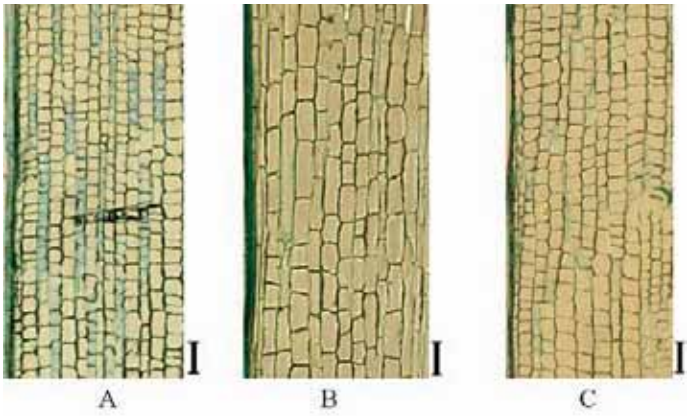

Figure 2 Longitudinal structure of neck internodes. **(A)** Aijiaonante, **(B)** CHA-1, **(C)** Tehuazhan.

Results

Characteristics of the dwarf mutant rice CHA-1

The internodes of the dwarf mutant rice CHA-1 were measured, and were all found to be shorter than those of Tehuazhan and Aijiaonante

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(had semi-dwarf gene *sd-1*). But the proportion of every internode elongation was normal and therefore, CHA-1 could be classified as dn-type dwarf mutant rice (**Fig. 1**).

Internode elongation is caused by cell division in the intercalary meristem, followed by cell elongation in the elongation zone. Therefore, dwarfing could be the result of a defect in one or both of these processes. To distinguish between these possibilities, the neck internodes from adult plants were examined under the microscope. **Figure 2** shows the cell morphology of the neck internode in CHA-1**,** Tehuazhan and Aijiaonante**.** CHA-1 had the shortest internode and longest internode cells. The result showed that the shortened internodes of CHA-1 could be due to the decreased number of internode cells.

Genetic analysis of height in CHA-1

In order to investigate the inheritance of the dwarf genes in CHA-1, crosses were carried out between CHA-1 with the original variety Tehuazhan and variety Aijiaonante (had semi-dwarf gene *sd-1*). Their F_2 segregated populations were used for genetic analysis. The ratio of high progeny to dwarf progeny was approximately 3:1, showing that their segregate patterns were according to one major gene, and that the dwarfism was not allelic to *sd-1*.

Furthermore, an F_2 population was constructed from the cross between CHA-1 and the tall variety Huiyangzhenzhuzao (with the homologous dominant gene *SD-1*). The plant height at ripening stage of each plant in theF_2 population was tested, and the data was collected to make a frequency distribution histogram of plant height (**Fig. 3**) and to perform an analysis by chi-square (χ^2) test (**Table 1**).

From **Figure 3** we can see the distinct separation of the tall and dwarf progeny in the F_2 population, and that the plant height distribution presented two continuous kurtosis, which also showed the pattern of segregation of the major gene. The ratio appeared to be 1.58 tall: 1dwarf, but not 3:1 or 15:1. Theoretically, CHA-1 was crossed with the tall variety with homologous dominant gene *SD-1*. Three possibilities would occur to in its F_2 population:

1. If the new gene and *sd-1* were on the same chromosome but not interacting, the segregation ratio of the F_2 population would be 3 tall: 1 dwarf;
2. If the dwarfism phenotype was controlled by these two recessive genes without linkage, then the segregation ratio of the F_2 population would be 9 tall: 6 semi-dwarf: 1 dwarf;
3. If *h* and *sd-1* were linked with complementary interaction, and they were controlling the dwarfism phenotype together, any gene that became homologous would cause dwarfism. Then, the segregation ratio of F_2 population would be among 3: 1 and 9: 7 (1.29: 1).

According to the results above, it can be assumed that the mutation gene *h* from CHA-1 was linked with *sd-1* to a certain extent. The two genes had complementary interaction. Therefore, the genotype of CHA-1 was *hhsd-1sd-1*.

Table 1. Segregation pattern of the F_2 population from the cross of CHA-1 and huiyangzhenzhuzao

Cross	Plant height packet		Actual proportion	Theory proportion	χ^2
CHA1/ huiyangzhenzhuzao	high plant	dwarf plant	1.58:1	3:1	9.76
	617	391		9:7	102.23

$\chi^2_{0.01}$=6.63, $\chi^2_{0.05}$=3.84, v=1

Mapping of dwarfing gene in CHA-1

To map the dwarf gene *h* locus, the mapping population consisted of the F_2 population from the cross between CHA-1 and semi-dwarf variety 02428 (which had the homologous dominant gene sd-1). Firstly, the DNA polymorphisms of two parents CHA-1 and 02428 were analyzed by using more than 200 pairs of rice SSR primers, which are distributed throughout rice chromosomes. Then, the SSR markers which showed various polymorphisms amongst the two parents, were filtrated. Secondly, the tall gene pool was built by blending the equivalent DNA of 10 tall individuals from the F_2 population. The dwarf gene pool was built in the same way. Thirdly, the polymorphism markers filtrated above were used to analyze the tall and dwarf gene pool (compared to the parents), then the marker RM302 on the long arm of rice chromosome 1 was found to have a linkage relationship with dwarf gene h. So, RM302 was used to detect the dwarf individuals from the F_2 population, whereby 12 recombinants were found in the 128 dwarf individuals. By analyzing the recombination individuals the dwarf gene h was finally mapped at a genetic distance of 4.715 cM from RM302 and of 5.915 cM from sd-1 (**Fig. 4**).

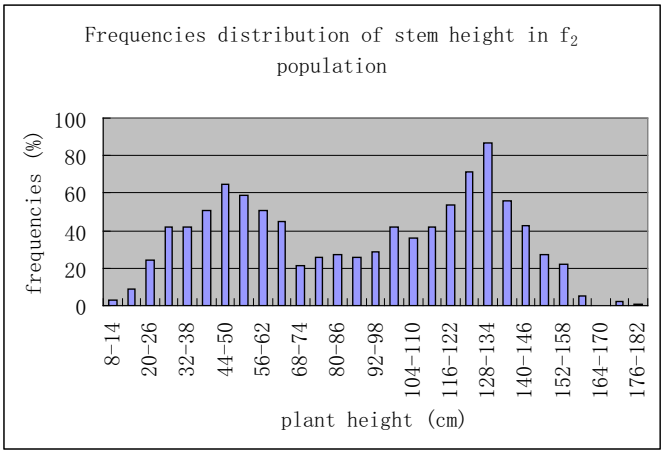

Figure 3 Frequency distribution histogram of plant height in the F_2 population from the cross of CHA-1 and Huiyangzhenzhuzao.

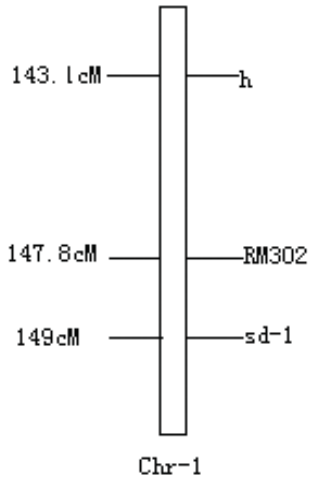

Figure 4 The genetic location of RM302, *h* and *sd-1* on chromosome 1.

Discussion

To date, the dwarfing genes *sd-1*, *d2*, *d18* (*d25*), *d10* (*d15*, *d16*) and *d61* were located in the rice chromosome 1. In this paper, results of genetic analysis and dwarfing gene mapping of CHA-1 have shown that *h* and *sd-1* were not allelic. In addition, *d2* and *d18* (*d25*) were abnormal dwarfing genes. Gene *d2* had a linkage genetic distance of 15.6 cM to molecular marker Npb359[3], and *d18* (*d25*) had a linkage genetic distance of 4.1

cM to molecular marker Npb96 [2]. Gene *d10* (*d15*, *d16*) was a dwarfing gene with many panicles, mapped to the short arm of chromosome 1, with linkage to the molecular marker RG462 [4]. And *d61* was mapped to the long arm of chromosome 1, with tight linkage to the RFLP marker C1370 [5]. Which indicated that *h* was not allelic to the dwarf genes above. So, we could confer that *h* was a new dwarf gene in rice.

This study and identification of the new dwarf gene *h* from CHA-1 will enrich the variety of dwarf rice resources. At the same time, the results obtained in this research laid a theoretical foundation for the application of the new dwarf mutant CHA-1 in rice breeding.

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Genetic Analysis and Mapping of Mutant Dwarf Gene *iga-1* in Rice

Z Chen*, T Guo, H Wang, J Zhang & D Rao

Abstract

The rice material Hangai-1 that is studied here is a stable dwarf mutant obtained by space mutation of rice cultivar Texianzhan 13(*indica*). Genetic analysis showed that its dwarf trait was controlled by two recessive semi-dwarf genes, *sd1* and a new semi-dwarf gene, named *iga-1*. This new semi-dwarf gene *iga-1* was located between microsatellite markers RM6645 and RM3837 on chromosome 5, the genetic distances between them were 0. 07cM and 1.21cM, respectively. The *iga-1* gene is possibly a multiple allele to the *d-1* gene. The semi-dwarf mutant with the new semi-dwarf gene *iga-1* was found to be insensitive to gibberellin 3(GA_3).

Introduction

Application and widespread adoption of dwarf rice varieties was one of the greatest achievements in rice breeding in the 20th century. However, there were only a few kind of dwarf sources used in rice breeding, and genetic analysis revealed the dwarf genes of these sources were mainly limited to *sd-1* and its alleles [1]. It is well known that the narrow background of variation and frequent use of single gene sources might become a potential bottleneck for crop breeding [2]. Therefore, exploiting, identifying and utilizing new useful semi-dwarf genes is an important subject for practical rice breeding. Space mutation has exhibited a wide application prospect in breeding [3-6]. In this study, the dwarf genetic characteristics and molecule mapping of Hangai-1 were analyzed, and the new rice semi-dwarf gene *iga-1* was identified from Hangai-1.

Materials and Methods

Dwarf genetic analysis of Hangai-1

Hangai-1 was crossed with a tall rice variety (Huiyangzhenzhuzao) and three semi-dwarf varieties (Texianzhan13, Aijiaonante, 02428) with semi-dwarf gene *sd-1* in the autumn of 2006. The parent, F_1 progenies and F_2 generation were planted in 2007.

Gene Mapping of the *iga-1* gene

A cross was made of indica variety Hangai-1 and japonica variety 02428 in the autumn of 2006. The parent, F_1 progeny and F_2 population were planted in 2007. The 912 F_2 dwarf population was selected as mapping population.

Insensitive to exogenous gibberellin GA_3 of the iga-1 semi-dwarf mutant

Seedling sensitivities of the iga-1 semi-dwarf mutant, Hangai-1, Aijiaonante, Texianzhan13 and Huiyangzhenzhuzao to 5mg/L, 10mg/L, 20mg/L, 30mg/L, 40mg/L gibberellin 3(GA_3) were investigated.

Results

Dwarf genetic analysis of Hangai-1

Hangai-1 was crossed with a tall rice variety (Huiyangzhenzhuzao) and three semi-dwarf varieties (Texianzhan13, Aijiaonante, 02428) with semi-dwarf gene *sd-1*. The F_1 progenies were as tall as the taller parent (Table 1), the dwarf trait of Hangai-1 being controlled by recessive nuclear gene. The segregation of the F_2 generation of Hangai-1 and Huiyangzhenzhuzao appeared to be 9 tall: 6 semi-dwarf: 1 dwarf, which indicated that the dwarfism of Hangai-1 was controlled by two recessive semi-dwarf genes without linkage (Table 2). The allelism test showed that one semi-dwarf gene of Hangai-1was allelic to *sd-1* gene and the other was a new one, nonallelic to *sd-1* gene and named as *iga-1*. The two recessive semi-dwarf genes have similar genetic effect and accumulated effect exists.

Table 1. Plant height of the parents and their F_1 hybrids.

Cross P_1/P_2	Plant height cm				F_1-MP
	P_1	P_2	F_1	MP	
Hangai-1/Huiyangzhenzhuzao	52.8±0.9	128.6±1.3	136.5±2.1	90.7	45.8
Hangai-1/Texianzhan13	52.8±0.9	114.1±1.5	115.3±2.4	83.5	31.8
Hangai-1/02428	52.8±0.9	97.3±1.3	121.2±1.8	75.3	45.9
02428/ Hangai-1	97.8±1.0	52.8±0.9	116.3±1.2	75.3	41.0

Table 2. Segregation of plant height in F_2 populations derived from the crosses between Hangai-1 and Texianzhan 13, Huiyangzhenzhuzao, respectively.

Cross	Plant height packet			Actual proportion	Theory proportion	χ^2
	High plant	Semi-dwarf plant	Dwarf plant			
Hangai-1/Texianzhan13		813	237	3.43:1	3:1	3.4
Hangai-1/Huiyangzhenzhuzao	872	524	104	8.38:5.04:1	9:6:1	4.7

Note: $\chi^2_{0.01}$=9.21, $\chi^2_{0.05}$=5.99, v=2; $\chi^2_{0.01}$=6.63, $\chi^2_{0.05}$=3.84, v=1

Gene Mapping of a Mutant Dwarf Gene iga-1 in Hangai-1

To map the *iga-1*, an F_2 population derived from a cross between Hangai-1 and 02428 was constructed. Using bulk analysis with SSR markers, the *iga-1* gene was located on the 58.77cM of rice chromosome 5 between two microsatellite markers RM6645 and RM3838, with genetic distance of 0.07cM and 1.21cM, respectively (**Fig. 1, Fig. 2**).

Figure 1 Linkage analysis between the semi-dwarf gene *iga-1* and microsatellite marker RM6645

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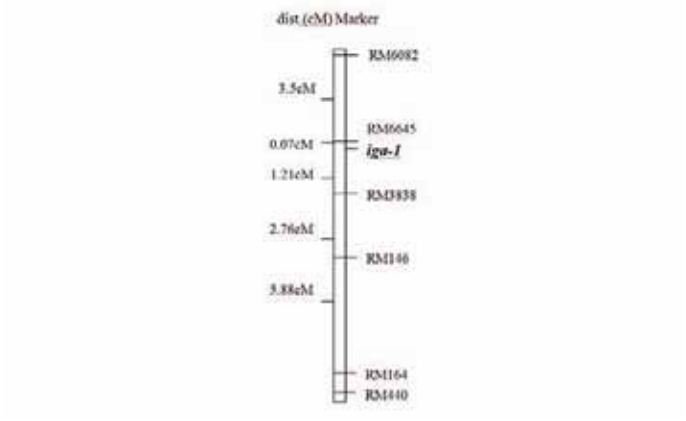

Figure 2 Fine location of the semi-dwarf gene *iga-1*. **1:** Dwarf parent; **2:** Crossing-over plant; **3:** Tall parent.

Insensitive to exogenous gibberellin GA3 of the *iga-1* semi-dwarf mutant

The *iga-1* semi-dwarf mutant was insensitive to exogenous gibberellin GA_3, and its wild phenotype of plant height was partially restored by exogenous GA_3. Moreover, the sensitiveness of *iga-1* was weaker than that of *sd-1*. (**Fig. 3**)

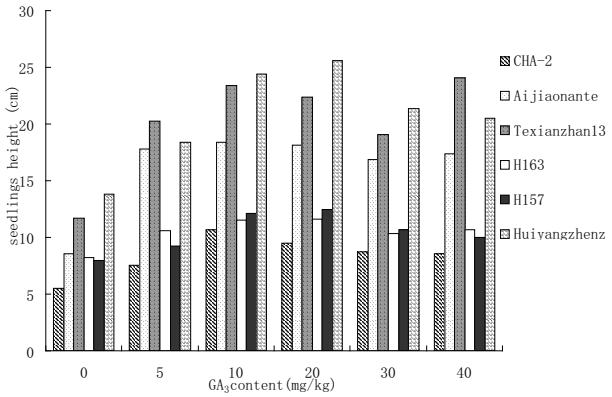

Figure 3 Height of seedling plants in response to GA_3.

Discussion

This research shows that the dwarf gene *d*-1,*d-1*^{x},*sd-7*,*sd-g* and *sd-n* were located on chromosome 5 [7]. The *d-1* gene was located on the 59cM of chromosome 5. The *d-1*^{x}gene is possibly an allele to the *d-1* gene and a multiple allele to the *sd-n* gene [8]. The *sd-7* gene was linked to the *d-1* gene [9]. The *sd-g* was linked to the *sd-n* gene [10]. In this study, the *iga-1* gene is possibly a multiple allele to the *d-1* gene. More research on it is underway.

Dwarf mutants have a very important effect on elucidating the regulation mechanism of stem development and plant breeding. This research showed that gibberellin (GA) has a close relationship with the dwarf mutant. Approximately all of the genes involved in the biosynthesis of the biologically active GA have now been isolated, using different kinds of strategies. However, research of the GA signal transduction is scanty and must be strengthened. The development of this kind of dwarf mutants involved in gibberellin signal transduction pathway was summarized. This work will help understand gibberellin signal transduction pathway, while this development can be used for the theoretical study of gibberellins in plant production.

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Selection of Wheat Mutant Genotypes Carrying HMW Glutenin Alleles Related to Baking Quality by Using PCR (STS method)

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Abstract

This study was performed in the Agriculture, Medicine and Industry Research School, Nuclear Science and Technology Research Institute of Iran in 2005-2006, through Polymerase Chain Reaction by using Sequence Tagged Site (STS) method, to characterize in terms of bread quality of some wheat mutant genotypes (Roshan, Omid, Tabasi, Azar and Azadi), their parents and other cultivars such as Chamran, Enia, Bezostaya, Tajan, Pishtaz and Chinese spring. Twelve pairs of primers were used in this study; seven of them were extracted from the literature and the others were designed from the D genome subunites sequences of wheat. Some studies on drought resistance, salt resistance, etc., have been done for these mutant genotypes, some of them showing good results. However, their baking quality has not been studied before. The alleles Dx2+Dy12 (with negative effect on bread quality) and Dx2*, Dx5+Dy10 (with positive effect on bread quality) had the main effect on wheat bread quality. Special primers of these subunits were used to amplify these alleles. Except for the cultivars that had Dx5+Dx10, six mutant genotypes whose parents did not have these alleles (T-66-58-60, Ro-5, Ro-4, Ro-3, Ro-1 and O-64-1-10), showed Dx5+Dx10. SDS-PAGE analyses showed no contradictory results with molecular experiments. Significant differences were seen on protein percentage for polymorphic mutant genotypes, Ro-1 , Ro-3 and Ro-5 with Roshan (their parent), at 1% probability level.

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops in the world and baking quality is an increasingly important trait, since it defines the uses and marketability of wheat products. Although the protein fraction of wheat seed known as gluten is associated with baking quality, High Molecular Weight (HMW) glutenins are the subunits most closely associated with this trait. These proteins have conserved amino and carboxy domains with Cys residues and a repetitive hydrophobic central domain. The homologous loci *Glu*-A, *Glu*-B and *Glu*-D control the synthesis of HMW glutenins and are found on linkage group 1 of their respective homologous genomes in wheat. Each loci encodes two subunits of different molecular weights, *x* and *y*. These subunits present a tight genetic linkage and are frequently reported as the *x* + *y* pair. Several independent studies report the close association between the allelic pair HMW*x*5+*y*10 at the locus *Glu*-D1 and improved baking quality, whereas the opposite applies to the allelic pair HMW*x*2+*x*12 [1,2]. Also, it has been demonstrated that good bread making quality is firmly associated with the presence of *Glu*-A1*x*2*. Moreover, *Glu*-A1*x-Null* and *Glu*-D1 *x*2+*y*12 were associated with small sedimentation volumes [3]. The nucleotide sequences of the alleles *Glu*-A1 *x*1 and *x*2* have been reported [4, 5]. The sequences of genes *Glu*-A1*x*2* and *Glu*-D1*x*5 were analyzed comparatively [4], showing that these genes have a similar structure and a high similarity. Recently, many authors used PCR-based analysis to distinguish between cultivars with different HMW glutenin subunits [6, 7, 8, 9]. Also, other genetic markers based on the PCR method as RAPDs and AFLP were used to identify polymorphism in DNA, and for practical use in recovering the genetic background of the recurrent parent during backcrossing in plant breeding programs. This paper reports the application of specific markers (STS method) as one of the best methods to amplify the coding sequence of each specific allele of the *Glu*-A1 and *Glu*-D1 genes that are related to baking quality.

Table 1. PCR cycling conditioned for the amplification of specific alleles of genes Glu-A1 and Glu-D1

Final Extension		Extension		Annealing		Primers
Time	Temp	Time	Temp	Time	Temp	
6’	72	1’	72	1’	64	P1P2
6’	72	1’	72	1’	65	P3P4
6’	72	1’	72	1’	66	P5P6
7’	70	2.20”	70	1’	58	P7P8
7’	70	2.30”	70	1’	66	P9P10
7’	70	2.10”	70	1.40”	60	P11P12
7’	70	2.30”	70	1’	61	P13P14
7’	72	2’	70	1’	61	P15P16
7’	70	1’	70	1’	59.5	P17P18
7’	70	2’	70	50”	62	P19P20
7’	70	2’	70	50”	60	P21P22
7’	70	3’	70	1’	59.5	P23P24

Table 2. Primer sequences used to amplify HMW glutenin subunits.

Forward PCR primers (5’.....3’)	Reverse PCR primers (5’.....3’)
P1 = 5’ ACCTTATCCATGCAAGCTACC 3’	P2 = 5’ CATGGCAGCCGACCGGCCAAC 3’
P3 = 5’ GTTGGCCGGTCGGCTGCCATG 3’	P4 = 5’ TGGAGAAGTTGGATAGTACC 3’
P5 = 5’ GCCTAGCAACCTTCACAATC 3’	P6 = 5’ GAAACCTGCTGCGGACAAG 3’
P7 = 5’ AGCCTAGCAACCTTCAC 3’	P8 = 5’ AGACATGCAGCACATACC 3’
P9 = 5’ CTAACTCGCCGTGCACA 3’	P10= 5’ AGCTAAGGTGCATGCATG 3’
P11= 5’ CTCGTCCCTATAAAAGCCTAGT 3’	P12= 5’ GAGACATGCAGCACATACT 3’
P13= 5’ AGCTAAGGTGCATGCATG 3’	P14= 5’ CTAACTCGCCGTGCACA 3’
P15= 5’ ATGGCTAAGCGGTTAGTCCTC 3’	P16= 5’ GCATTGTCGGCCAGCCAGTGA 3’
P17= 5’ GCATTGTCGGCCAGCCAGTGA 3’	P18= 5’ ACAAGGGCAACAAGGTCAGCA 3’
P19= 5’ GCATTGTCGGCTAGCCAGTGA 3’	P20= 5’ ATGGCTAAGCGGCTGGTCCTC 3’
P21= 5’ ATGGCTAAGCGGTTAGTCCTC 3’	P22= 5’ ACAAGGGCAACAAGGTCAGCA 3’
P23= 5’ ATGGCTAAGCGGCTGGTCCTC 3’	P24= 5’AGGACAAGGGCAACAGTCAGG 3’

Materials and Methods

Genomic DNA was extracted by CTAB method from 6-8 g of fresh leaves from Tabasi mutants (T-65-9-1P, T-66-58-6, T-66-58-9, T-65-6, T-65-5-1, T-66-58-10, T-66-58-12, T-65-4, T-65-9-1, T-67-7-1, T-58-8, T-58-7, T-66-I-II, T-67-60, T-58-14, T-65-9-II-4 and T-66-58-60), Omid mutants (O-64-1-1, O-64-4 and O-6-1-1), Roshan mutants (Ro-1, Ro-2, Ro-3, Ro-4, Ro-5, Ro-6, Ro-7, Ro-8, Ro-9, Ro-10, Ro-11 and Ro-12), As-48 (Azadi mutant), Azar mutant with their parents (Tabasi, Omid, Roshan, Azadi and Azar), and some other cultivars like Chamran, Navid, Atrak, Tajan, Inia, Bezostaya, Tajan-e-garm, Pishtaz and Chinese spring,

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whose baking quality and alleles are known. The 25 µl amplification reaction contained PCR Buffer 1x (2.5µl), $MgCl_2$ 2mM, dNTPs 5mM, Primers (10+10mM), *Taq* DNA polymerase (1.5unit), DNA (250ng), dd H_2O (up to 25 µl). All materials except the primers were from Fermentas, *Taq* DNA polymerase was Native, without BSA, and primers were from Biotech (see **Tables 1 and 2**).

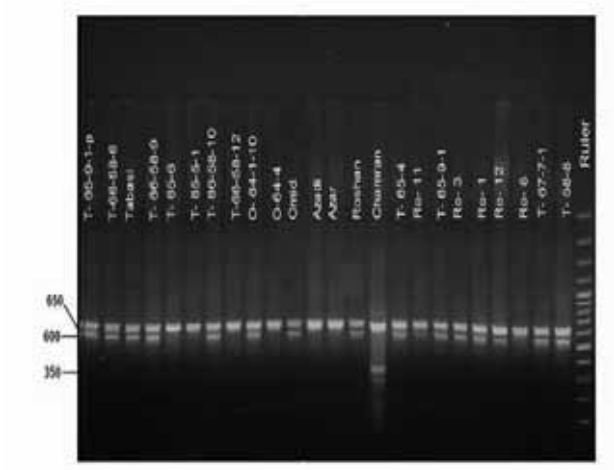

Figure 1 Agarose gel (1.7%) for P1P2 (Dy10 and Dy12).

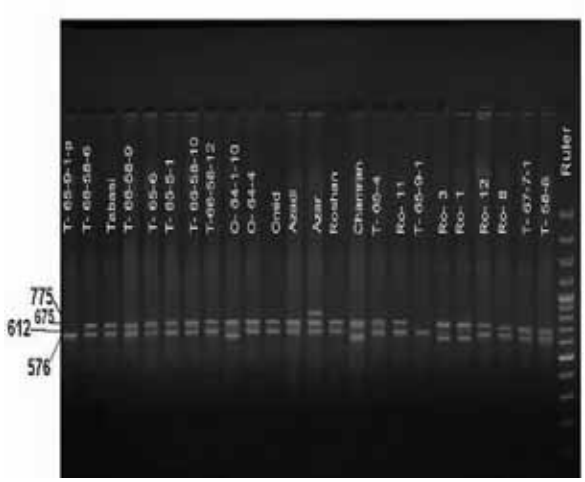

Figure 2 Agarose gel (1.7%) for P3P4 (Dy10 and Dy12).

Results and Discussion

There is a genetic association between baking quality and HMW glutenin allelic composition in wheat [10]. In genome A and B there are also HMW loci and their allelic richness precludes the development of gene-specific detection systems. But there are only two reported alleles in genome D that are HMW loci.

Developments in DNA marker technology and use of such markers in marker assisted selection provide new solutions for selecting and maintaining desirable genotypes, while using PCR analysis with specific primers is good for identifying specific genes and nearly identical alleles as well. Also, SDS-PAGE method is one of the most widespread methods for detecting allelic forms related to good or poor quality in baking. Shewry, *et al* [10] reported that the mobility of HMW glutenin subunits in this method is not always being correlated to their molecular weights, and this could be a big problem for breeding programs. Marker assisted selection can help to avoid this misleading interpretation of SDS-PAGE results. Some mutations cannot be detected with SDS-PAGE systems and would therefore go unnoticed, resulting in a mistaken selection program. Discriminating between DNA samples that carry poor or good baking quality alleles is very simple and can achieved in less than four hours. Also it can eliminate the hazardous reagents such as acrylamide. Another advantage of using this system is the possibility to use any part of the plant, which is faster than having to wait for availability of seed tissue.

Primers P1P2 and P3P4 were extracted from [11]. P1P2 amplifies Dy10 (650 bp) and Dy12 (650 bp). This primer showed two bands other than the single one (600 bp) reported in [11], and it was not a specific band for Dy10 or Dy12 but another band that did not exist in the Smith, *et al.* study [11], at 350 bp and seen only in Chamran. P3P4 amplifies Dy10 (576 bp) and Dy12(612 bp). This primer also showed two bands instead of one (675 bp) as in [11], and it was a specific band for By9. All the genotypes had this allele, except Ro-9, T-65-9-1p and T-65-9-1 (despite the fact that their parents have it). Another band (775 bp) was only seen in Azar. **Figures 1 and 2** illustrate these results.

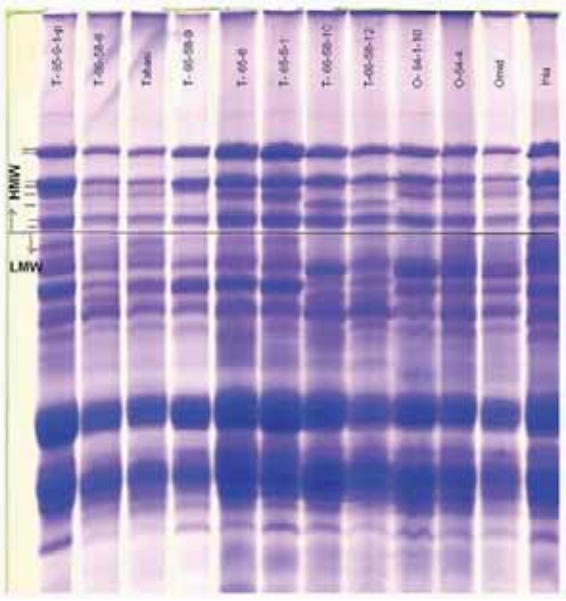

Figure 3 SDS-PAGE poly-acrylamid gel for seed storage protein.

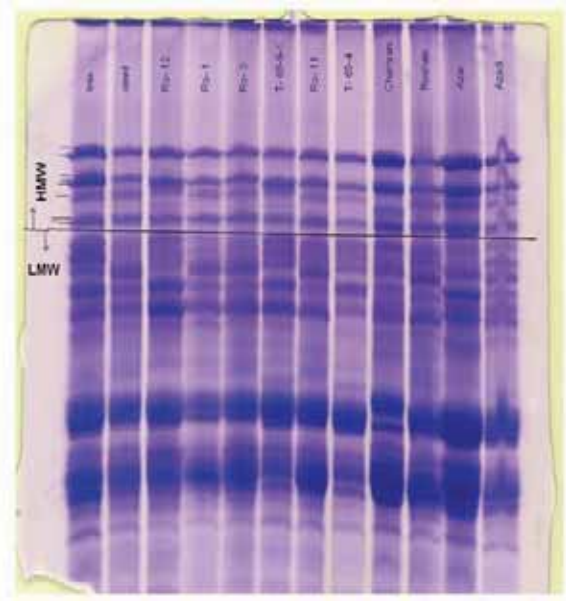

Figure 4 SDS-PAGE poly-acrylamid gel for seed storage protein.

Primer P5P6 that amplifies Dx5 (450 bp) was from [12,13], while primers P7P8, P9P10, P11P12 and P13P14 were from [9]. P7P8 amplifies Dx5 (2775 bp), but this band did not appear in spite of many changes performed to the method. However, there were two bands of 550 bp and 1350 bp, that were not specific for Dx5, and which appeared on all the genotypes. P9P10 amplifies Dy10 (2135 bp), P11P12 amplifies Dx2 (2799 bp) and P13P14 amplifies Dy12 (2190 bp). Other primers (P15 up to P24) were designed from the D genome subunit sequences. P15P16 should amplify Dx5 (2520 bp), also P17P18, P19P20, P21P22 and P23P24 should amplify Dx2.1 (651 bp), Dy10 (1947 bp), Dx2.1 (1876 bp) and Dy12 (1947 bp) respectively, but none of these bands appeared.

Our results showed that the best primers for Marker Assisted Selection are P3P4 and P5P6. P3P4 is the best because obtaining specific bands in this primer is easier and it also shows three specific bands for Dy10, Dy12 and By9.

This study shows that six mutant genotypes (T-66-58-60, Ro-1, Ro-3, Ro-4, Ro-5 and O-64-1-1) carry alleles alike the genotypes that have good baking quality (Chamran, Tajan, Bezostaya, etc), although their parents do not have these alleles.

Protein percentage for mutant genotypes (**Table 4**) showing polymorphism, as in Ro-1 , Ro-3 and Ro-5 were different from Roshan (their parent) at 1% probability level.

In three genotypes (T-65-9-1, T-65-9-1p and Ro-9,) mutation caused a change in B*y*9 sequencing.

P3P4 primers were the best primers when considering three alleles at the same time and the bands that appeared were sharper than the others (maybe because of the size of these bands). Also, using multiplex PCR is recommended in the situation where there are bands of different sizes.

Thus, in order to validate the results of molecular tests, the SDS-PAGE method was undertaken, with results as shown in the gels of **Figures 3 and 4**.

Table 3. Summary of protein percentage (CV=2%) analysis

(Ms)	(S.O.V)	(Df)
Genotype	10	5.3269**
Error	22	0.05

Table 4. Comparing of protein mean by Duncan's multiple-range test

Genotype	Mean protein percentage	Ranking
Ro-3	13.3	A
Roshan	12.6	B
Ro-4	12.3	B
Ro-5	12	C
Ro-1	11.8	C
Omid	10.6	D
O-64-10-10	10.6	D
Azar	10.2	E
T-65-9-1P	9.8	F
T-65-58-60	9.8	F
Tabasi	9.3	F

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Isolation and Characterization of Retrotransposons in Wild and Cultivated Peanut Species

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Abstract

Retrotransposons are considered a possible source for mutations due to their potential of spreading in the genome using a "copy and paste"-like mechanism. Here we report on the isolation and characterization of a new Ty3-*gypsy* retrotransposon from allotetraploid peanut (*Arachis hypogaea,* 2n=4x=40) and its diploid ancestors *A. duranensis* (AA-genome, 2n=20) and *A. ipaënsis* (BB-genome, 2n=20). We have identified two repetitive sequences, one showing high similarity at the amino acid level to the reverse transcriptase of *Athila*-type retrotransposons, the other being AT-rich with no similarities to gene bank sequences. Results from genome walking experiments gave first evidence that both sequences represented parts of the same Ty3-*gypsy* retrotransposon, the 5'-LTR (long terminal repeat)- and the *pol* (polyprotein)-region respectively. Fluorescent *in situ* hybridization (FISH) experiments showed that the element is dispersedly distributed on the chromosomes, absent from centromeres and telomeric regions, and more prominent in chromosomes of the A-genome. The element appeared to be moderately repetitive with copy numbers of about 830 (*A. ipaënsis),* 2,600 *(A. duranensis),* and 3,000 (*Arachis hypogaea)* per haploid genome. Phylogenetic analysis of the deduced amino acid sequences of 80 isolated reverse transcriptase clones from the three species shed light on its evolution within the peanut species. The isolated sequences contained multiple stop-codons and so far, no evidence has been found that the element is still active. An outlook is given regarding finding new tools for the advancement of *Arachis* breeding programs aimed at the transfer of resistance to biotic and abiotic stresses to peanut.

Introduction

Cultivated peanut *(Arachis hypogaea)* is an allotetraploid species (AABB genome, 2n = 4x = 40), which derived from a single hybridization event between two diploid *Arachis* species and subsequent spontaneous chromosome doubling. Recent studies including those based on fluorescent *in situ* hybridization using rDNA sequences as probes suggest that *A. duranensis* (AA, 2n = 20) and *A. ipaënsis* (BB, 2n = 20) are the most probable ancestors [1, 2]. Its low genetic diversity is the major bottleneck that hampers crop improvement programs and genetic studies in peanut. In contrast, most wild *Arachis* species are diploid with high genetic diversity, and moreover, some are a source for resistance to important biotic and abiotic stresses. In order to elucidate the relationships of wild and cultivated peanut genomes, thereby improving our ability to efficiently introgress wild genes into the peanut crop, repetitive elements, especially retrotransposons, are an interesting research object.

Retrotransposons are mobile genetic elements present in many different organisms. Active elements replicate through a mechanism of reverse transcription and insertion of a new copy into new chromosomal sites, where they can cause mutations (for a recent review on their life cycle see [3]). Retroelements, particularly the LTR (long terminal repeat) retrotransposons, constitute the major part of repetitive DNA and contribute substantially to genome size [4, 5]. Based on phylogenetic analysis of their reverse transcriptase (*rt*) sequences and on structural differences LTR-, retrotransposons can be divided into two major lineages, one consisting of the Ty1-*copia* retrotransposons (*pseudiviridae*), and the other of the Ty3-*gypsy* retrotranspsons (*metaviridae*) [6, 7]. Retrovirus-like retrotransposons are characterized through an additional open reading frame (ORF) encoding transmembrane domains, which are characteristic for envelope (*env*) genes. Examples of retrovirus-like transposon families are the Ty3-*gypsy* retrotransposons *Athila* [8], *Cyclops* [9], and *Calypso* [10]. Several LTR-retrotransposons have been reported to be present in legumes. The first indication for presence of an LTR retrotransposon in peanut was given by Chavanne, *et al.* [9], who characterized the *gypsy*-like retrotransposon *Cyclops* in pea and detected hybridization of a fragment of its reverse transcriptase to genomic DNA of various legumes, including *A. hypogaea*. Yüksel, *et al.* [11] screened their BAC library of *A. hypogaea* and found a sequence with similarity to an *Arabidopsis copia* element.

Some elements seem to be constitutively expressed, for instance the *Ogre* element of pea [12]. Others are silent and can be activated upon certain stress signals such as tissue culture (*Tos17* retrotransposon in rice [13]), ionizing irradiation (Ty1 in *Saccharomyces cerivisiae* [14]), wounding (*Tto1* in tobacco [15]), or allopolyploidization (*Wis2-1A* in synthetic wheat allotetraploids [16]). The ability of some retrotransposons to become active again after stimulation makes them an ideal tool for inducing desired genetic variability, and also for reverse genetics approaches, as it was shown by the generation of about 50,000 rice insertion lines with *Tos 17* [17]. Furthermore, their abundance and ability to transpose make them good potential markers in form of the PCR-based techniques IRAP (inter-retrotransposon amplified polymorphism) and REMAP (retrotransposon-microsatellite amplified polymorphism) that detect retrotransposon integration events in the genome [18].

So far, with the exception of rDNA sequences [1] relatively little is known about the features and localization of repetitive elements in *Arachis* species. This applies especially to retrotransposons. Here we report on the isolation and characterization of a new *Athila*-like retrotransposon in *Arachis* species.

Materials and Methods

Plant materials and DNA extraction: Leaf and root tissue were obtained from *A. hypogaea* cv. Tatu, *A. duranensis* (accession V14167c), *A. stenosperma* (accession V10309), and *A. ipaënsis* (accession KG30076c). Plants were grown from seeds under greenhouse conditions. All plants were obtained from the Brazilian *Arachis* germplasm collection, maintained at Embrapa Genetic Resources and Biotechnology — CENARGEN (Brasília-DF, Brazil).

Cloning of reverse transcription sequences and phylogenetic analysis: To enable phylogenetic analysis of the retroelement in *A. hypogaea*, *A.*

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duranensis, and *A. ipaënsis* a set of three forward (F) and three reverse (F) PCR primers have been designed to amplify the total sequence of the *Athila*-like reverse transcriptase (*rt*):
Rep-RT-F1 5'-AAGGACACACAAGACAGCTC-3';
Rep-RT-F2 5'-GTACGCACAAGATCCTATTG-3';
Rep-RT-F3 5'-CTAAATCCAGCCATGAAGG-3';
Rep-RT-R1 5'-GTCAGCTACAAGGAGATTGC-3';
Rep-RT-R2 5'-GGAGATGATAGGTGCAGAAG-3';
Rep-RT-R3 5'-TCACACATCAGTTCAAATGG-3'.

Sequence analysis and construction of contigs has been done using the Staden Package software [19]. For multiple alignment of amino acid- and DNA sequences the program ClustalX [20] has been used. The results of alignments have been utilized for construction of phylogenetic trees using the program Mega 3.1 [21].

Genome walking: In order to extend sequence information into the flanking genomic regions of the two pseudo-contigs a modified Genome Walker™ strategy has been applied. Genomic DNA of *A. hypogaea* (1.5 µg) was digested with 15 units of the restriction enzyme *Pvu*II. After complete digestion the samples were purified with Phenol/Chloroform, precipitated and re-suspended in 20 µl $TE_{(0.1)}$. 4 µl of the digested was used for ligation of a *Pvu*II adaptor, consisting of 5'-ACTCGATTCTCAACCCGAAAGTATAGATCCCA-3' (long arm) and 5'-Phosphate-TGGGATCTATACTT-H_2N-3'. PCR reactions have been performed with the adapter primer AP1 (5'-ACTCGATTCTCAACCCGAAAG-3') and genome specific primers directed outwards the known sequences. For Rep-1 primer Rep-1-Out-Right-2 (5'- GTTGCCGGGGATTGTTC-3') was used to amplify sequences downstream, for Rep-2 it were the primers Rep-2-Out-Left (5'-AATCATGTCCTCAATTACGC-3') and Rep-2-Out-Right (5'-TGGTGACTGAAGGAATTGTC-3') for upstream and downstream amplification. PCR products have been cloned and sequenced using Sp6 and T7 sequencing primers.

Chromosome preparation and Fluorescent in situ hybridization: Root tips were collected from young plants in the greenhouse or from rooting leaf petioles that have been cultivated in humid petri dishes in an incubator with average temperature of 25° C [22]. Chromosome preparations have been made according to Maluszynska and Heslop-Harrison [23]. Pretreatment, hybridization, washing and detection procedures essentially followed protocols published by Schwarzacher and Heslop-Harrison [24] with post-hybridization washes at 83% stringency.

Results

Isolation and structure of the Arachis retroelement: Based on a dot blot survey of short insert libraries of *A. duranensis* and *A. ipaënsis* that were probed with genomic DNA of both species, several clones were identified resembling repetitive sequences. Using the sequences of these clones and additional gene bank sequences, two pseudo-contigs, Rep-1 and Rep-2, were established using the Staden-Package software [19]. A BLASTx search [25] revealed high similarities between one of those contigs (Rep-2) and the reverse transcriptase region of the Ty3-*gypsy* retrotransposon *Athila*. On the other hand, the Rep-1 contig was AT-rich with no similarities to any genic sequences in gene bank. Extended sequence information from both repeats was gained through a modified genome walker strategy, involving the ligation of specific adaptors to genomic restriction fragments and subsequent amplification of sequences adjacent to the repeat clones using adaptor- and repeat-specific primers. From 24 clones isolated from the right site of Rep-1, three had inserts of 1200 to 2000 bps (**Fig. 1**). Those three inserts showed significant similarity ($2e^{-32}$) to the *gag-pol* region of the Ty3-*gypsy* retrotransposons *Ogre* (pea) and *Athila* (*Arabidopsis*). Additionally, the new sequences resembled at their 5'-end the primer-binding site (PBS) of *Calypso*- and *Athila* like retrotransposons, which is complementary to the 3'-end of the Asp tRNA. Translated sequences received from Rep-2 genome walking experiments revealed presence of conserved amino acid motifs that are characteristic for the retrovirus-like Ty3-*gypsy* elements. These results gave first evidence that Rep-1 and Rep-2 are parts of the LTR- and the *pol* region of the same Ty3-*gypsy* retrotransposon. We aimed to obtain the full sequence of this element using the recently generated BAC libraries of the *A. duranensis* and *A. ipaënsis* genomes [26]. Using PCR screening and Southern hybridization, four BAC clones have been identified that contain at least one *rt*- and two or more LTR-fragments, which implies the possibility of isolation of a complete element. According to preliminary results from PCR cloning the total length of the element is bigger than 11,000 bps and does not contain an *env*-typical ORF.

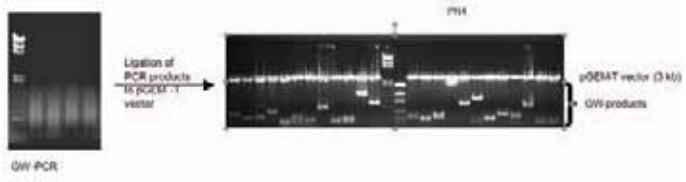

Figure 1 Cloning of sequences downstream of the isolated AT-rich repeat Rep-1. The translated sequence of clone PR4 revealed significant similarity ($2e^{-32}$) to the gag-pol region of the Ty3-gypsy retrotransposons *Athila* in *A. thaliana* and *Ogre* in pea. (GW: genome walking).

Copy Number Estimation: Copy number estimations have been made on the basis of non-radioactive dot blot hybridizations of a retrotransposon subclone against dilution series of itself and of genomic DNA from the three *Arachis* species. Subclone dilutions represented one to 100,000 copies of the retrotransposon sequence in 500 ng of genomic DNA. The strength of hybridization signals in the chemilumigraphs was quantified using the MultiGauge software (Fucifilm) and the slope of the regression curves of the resulting plots have been used for calculating the copy numbers. The representative *rt*-subclone Ah-9 was estimated to be present in the *A. ipaënsis* genome (2C = 2.8 pg) with about 830 copies (equivalent to 0.7% of the genome), in *A. duraensis* (2C = 2.61 pg) with about 2,600 copies (equivalent to 2.3% of the genome), and in *A. hypogaea* (2C = 5.93 pg) with 3,000 copies (equivalent to 1.2% of the genome) per haploid genome. (Genome sizes according to Temsch and Greilhuber [27, 28].)

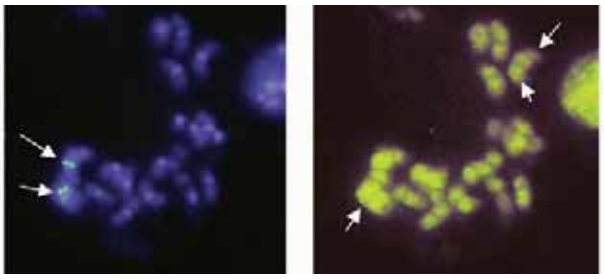

Figure 2 FISH of chromosome spread of A. stenosperma (A-genome). Left: hybridization with the wheat 5S-rDNA probe [29] reveals one sub-centromeric 5S-rDNA site (arrows). Right: re-hybridization with Dig-labeled LTR-probe. Note the lack of hybridization to centromeres and telomeric regions (arrows).

Chromosomal Distribution of retroelement: To determine the chromosomal distribution of the retrotransposon metaphase spreads of *A. duranensis*, *A. stenosperma* (A-genome), and *A. hypogaea* have been hybridized with Dig-labeled LTR- and reverse transcriptase probes. DAPI staining of the chromosomes revealed centromeric bands, which

are typical for the *Arachis* A-genome, whereas B-genome chromosomes do not show such bands [1]. Hybridization of A-genome chromosome spreads with the LTR probe showed dispersed distribution of the element in euchromatic regions of chromosomes and absence from heterochromatic regions, such as the centromer and telomeric regions (**Fig. 2**). When hybridized to metaphase spreads of *A. hypogaea*, the same probe appeared to be localized preferably in the A-genome of *Arachis,* which became obvious by comparison with the DAPI stained spreads allowing differentiation between A- and B- genome chromosomes. This kind of preferential hybridization to the A-genome was also detected when using a clone representing the reverse transcriptase as a probe (data not shown).

Phylogenetic Analysis: From 87 reverse transcriptase sequences isolated and cloned from *A. hypogaea*, *A. duranensis* , and *A. ipaënsis*, 80 have been selected for analysis of their evolution. Using Mega 3.1 the genetic distances between the sequences were calculated (**Table 1**). Three groups are shown, each of them contained all sequences of one species. The distances within each group and between the groups have been analyzed with regard to the nucleotide and as well the amino acid sequences. The data reveal that the DNA sequences isolated from *A. duranensis* suffered less modifications as compared to the ones from *A. hypogaea* and *A. ipaënsis*, which revealed the largest genetic distances. This trend is even more obvious when looking at the amino acid sequences, probably a result of changes in the reading frame that could have generated different amino acids.

Table 1. Genetic distances between DNA sequences and between amino acid sequences of isolated *rt*-genes from *A. hypogaea* (Ah), *A. duranensis* (Ad), and *A. ipaënsis* (Ai). The mean value corresponds to all 80 sequences of DNA and/or amino acids.

Groups	Distance between the sequences (%)	
	Seq. DNA	Seq. amino acids
Ah x Ah	8.68	18.83
Ad x Ad	8.20	17.44
Ai x Ai	8.95	19.87
Mean value	8,61	18.71

Discussion

The present work evolved from our efforts in isolating repetitive elements from the A- and B- genome of *Arachis* aimed at finding out more about the genomic relationships between the ancestors of cultivated peanut. The described element is the first characterized retrotransposon in *Arachis*. Preliminary sequencing results revealed that it belongs to the Ty3-*gypsy* retrotransposon family with high similarities to retrovirus-like elements, particularly to the *Athila* element from *Arabidopsis*. However, it also became clear that this new element has its own characteristics, which make it different from the related elements. FISH analysis has shown its localization in the euchromatic region of the chromosomes, and absence from centromeric and telomeric regions. This kind of distribution pattern was shown to be typical for Ty1-*copia* elements, such as BARE [30]. In contrast, the *Athila* element in *Arabidopsis* is associated with centromeric and pericentromeric regions [31]. We have detected preferential hybridization of the *Arachis* element to A-genome chromosomes. These results were substantiated by copy number estimations, where more than three times less copies were estimated for *A. ipaënsis* than for *A. duranensis*. Since multiple insertions of the element were found in *A. hypogaea*, *A. ipaënsis*, *A. duranensis*, and *A. stenosperma*, it can be assumed that it is an ancient component of the genus. In a phylogenetic analysis of 80 *rt*-sequences isolated from all three species, fewer mutations were found between the sequences derived from *A. duranensis* as compared to the ones from *A. ipaënsis*. We conclude that the element was present in the common ancestor of the *Arachis* wild species at low copy number. After differentiation it amplified in *A. duranensis*, whereas in *A. ipaënsis* the amplification was low, the copy number remained stable or was even reduced through elimination. We could not find evidence for activity of the element by screening all relevant EST databases. It appeared that all sequences isolated included stop codons. Using BAC sequences we are now on the way to a more detailed analysis of this element. Further efforts are directed towards identifying elements, which can be activated thus enabling their use for induced mutations approaches.

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Characterization of Resistance Gene Analogs in *Musa acuminata* Cultivars Contrasting in Resistance to Biotic Stresses

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Abstract

The majority of commercial banana cultivars (*Musa* sp.) have evolved via asexual vegetative propagation, with diversity dependent upon somatic mutation. Restricted variation has resulted in a crops with little resistance to pests and disease, and conventional breeding efforts are limited due to limited viable seed production. Numerous disease resistance genes (R-genes / R-proteins) have been characterized in plants, recognizing and conferring resistance to bacteria, virus, fungi and nematodes. The identification and cloning of R-genes in *Musa* would contribute to germplasm improvement. To date, five main R-gene classes have been identified, based upon protein domains, with the most abundant coding for nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins. Primers designed from conserved protein motifs have enabled amplification of NBS homologues across diverse plant species. In *Musa*, our group has identified over 50 distinct NBS-LRR type resistance gene analogs (RGAs) in the resistant wild diploid *M. acuminata* Calcutta 4. The aim of this work was to characterize RGAs in *M. acuminata* cultivars contrasting in resistance to Black leaf Streak Disease. PCR amplification was conducted using DNA from *M. acuminata* cultivars Calcutta 4 (resistant) and Pisang Berlin (susceptible). Degenerate primers targeted sequences homologous to the NBS-LRR R-gene family. Following sequencing and processing of cloned PCR products, 63 out of a total of 136 high quality sequences showed homology to R-genes or RGAs. Phylogenetic analysis was conducted on deduced amino-acid sequences. Degenerate primers were also developed targeting an R-gene family of cytoplasmic serine-threonine (Ser/Thr) receptor-like kinases (RLKs) with extracellular LRRs, for application across cultivars. Studies are also planned for selection and full length sequencing of clones from *M. acuminata* and *M. balbisiana* BAC libraries containing novel RGAs characterized in this study, as an approach for complete R-gene sequence characterization, applicable both in transformation and breeding programs for banana genetic improvement.

Introduction

Banana cultivars have evolved from diploid, triploid and tetraploid wild Asian species of *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). Wild species are generally seminiferous, whilst the majority of commercial cultivars grown today are sterile triploids, with fruit development via parthenocarpy. As these have evolved via asexual vegetative propagation, the genetic base is narrow, with diversity dependent upon somatic mutation. Such restricted variation has resulted in a crop with little resistance to pests and disease. Current breeding for resistance generally relies upon crosses between improved resistant hybrid diploids and commercial triploids, as well as evaluation of tetraploids [1]. Conventional breeding in *Musa* diploids and triploids is, however, hampered due to low numbers or a complete absence of seeds, caused by either a lack of viable pollen, or inefficient pollinating insects.

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Resistant plant genotypes prevent pathogen entry as non-host plants, or via activation of defense mechanisms following pathogen recognition by resistance genes (R-genes / R-proteins), leading to responses such as the hypersensitive response, synthesis of antimicrobials, cell wall thickening, and vessel blockage. Over the last 15 years, over 40 R-genes have been characterized from both model plants and important crop species [2], conferring resistance to bacteria, virus, fungi and nematodes. The identification and cloning of R-genes in the *Musa* genome would contribute significantly to the future improvement of banana germplasm. To date, five R-gene classes have been identified in plants, based upon conserved protein domains. The most abundant class is the cytoplasmic nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins. Other classes comprise proteins with extracytoplasmic LRRs (eLRRs) anchored to a transmembrane (TM) domain (receptor-like proteins [RLPs]), cytoplasmic serine-threonine (Ser/Thr) receptor-like kinases (RLKs) with extracellular LRRs, cytoplasmic Ser/Thr kinases without LRRs, and proteins with a membrane anchor fused to a coiled coil (CC) domain. The common NBS-LRR-encoding proteins currently include over 20 proven R-genes from diverse plant species. Primers designed from conserved motifs have also been used to amplify NBS homologues from numerous different plant species (for review see [3]). In the case of *Musa*, our group has identified over 50 distinct NBS-LRR type resistance gene analogs in the resistant wild diploid *M. acuminata* Calcutta 4 [4].

The objective of this work was to continue characterization of RGAs in *M. acuminata* cultivars contrasting in resistance to Black Leaf Streak Disease (BLSD), caused by the ascomycete *Mycosphaerella fijiensis*. Additional degenerate primers were also designed, targeting R-gene cytoplasmic serine-threonine (Ser/Thr) receptor-like kinases (RLKs) with extracellular LRRs, for application across cultivars.

Materials and Methods

DNA extraction

Genomic DNA was extracted from *M. acuminata* cultivars Calcutta 4 (resistant to BLSD) and Pisang Berlin (susceptible to BLSD), using a standard CTAB approach [5], with 200mg of young leaf tissue macerated on a Bio 101 Thermo Savant FastPrep® FP 120 cell disrupter (Qbiogene, Irvine, CA, USA).

PCR amplification

Degenerate Primers: Two degenerate primers were tested for amplification of RGAs in the *Musa* cultivars. These were previously designed in a study by our group from conserved motifs in non-TIR NBS-LRR domain-containing monocotyledon sequences, with proven efficiency in amplification of RGAs in *M. acuminata* Calcutta 4 [4] (**Table 1**).

PCR amplification

PCR reactions were performed in 25 µl volumes, containing 50 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, 1.25 U of Platinum Taq polymerase (Invitrogen, Brazil), and 1X Taq polymerase buffer (Invitrogen, Brazil). Amplification was conducted

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with the following temperature cycling program: 96°C for 5 min; 35 cycles of 96°C for 1 min, 45°C for 1 min, and 72°C for 1 min; plus an extra elongation period of 10 min at 72°C.

Cloning and sequencing

Following electrophoresis, PCR products of expected size were purified using a Qiagen QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Products were cloned using pGEM-T-Easy (Promega, Madison, WI, USA). Ligations were desalted using Millipore dialysis membranes (0.02 μM) and DH5α *Escherichia coli* cells were transformed by electroporation using a GenePulser II (Bio-Rad, Hercules, CA, USA) at 2.5 kV and 200Ω. Plasmid DNA was extracted from recombinant clones by a standard alkaline lysis procedure [6]. Single pass sequencing of clones was conducted on ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA), using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA).

Sequence analysis

Vector and poor quality sequences were removed using the Staden sequence analysis software package [6]. Contig assembly was performed using CAP3 [7]. RGAs were identified based upon sequence similarity using BLASTX [8], against a local database of *A. thaliana* R-genes and homologues, as described in [9]. Derived protein sequence alignments were obtained using the program MUSCLE [10] and included representative non-TIR NBS-LRR sequences from *A. thaliana* and *O. sativa*. Phylogenetic inference was performed using the program Mega v.4.1 [11], via maximum parsimony with 500 bootstrapping replicates.

Degenerate primer design for cytoplasmic serine-threonine (Ser/Thr) receptor-like kinases (RLKs) with extracellular LRRs

Degenerate primers were designed using an adaptation of an *in silico* protocol described by [4]. The process began with HMMER-based selection of sequences from GenBank containing the characteristic domain shared by this class of R-genes, visualized using the Conserved Domain Database, v2.14. Sequences were aligned in MUSCLE [10], and after redundant sequence removal (95% identity threshold), five subgroups were arranged, based upon similarity level using the program CLUSS [12]. All conserved motifs identified served as candidates for degenerate primer design, conducted using the program CODEHOP [13].

Results and Discussion

NBS-LRR RGA isolation

The primer combination applied in this study (3F2–13R1) consistently amplified products of approximately 650 bp in size in both cultivars Calcutta 4 and Pisang Berlin. A total of 136 high quality sequences were generated from insert-containing recombinant plasmids for these two cultivars, of which 63 showed significant similarity to known *A. thaliana* R-genes and homologues (E-value $\leq 10^{-5}$), based upon searches using the BLASTX program. All *Musa* RGAs conformed to the non-TIR NBS class, with a final tryptophan residue in the kinase 2 motif, as expected, given their apparent absence in monocotyledonous genomes [14].

Table 1. Degenerate primer sequences and target motifs used for RGA isolation in *M. acuminata* cultivars

Degenerate Primer	Target motif name / plant origin	Primer sequence (5' to 3')[a]	Author
3F2 (forward)	Kinase 2 / monocotyledon	GAGGTACTTCCTGGTGCTGgaygayrtbtgg	[4]
13R1 (reverse)	LRR / monocotyledon	CGGCCAAGTCGTGCAyvakrtcrtgca	[4]

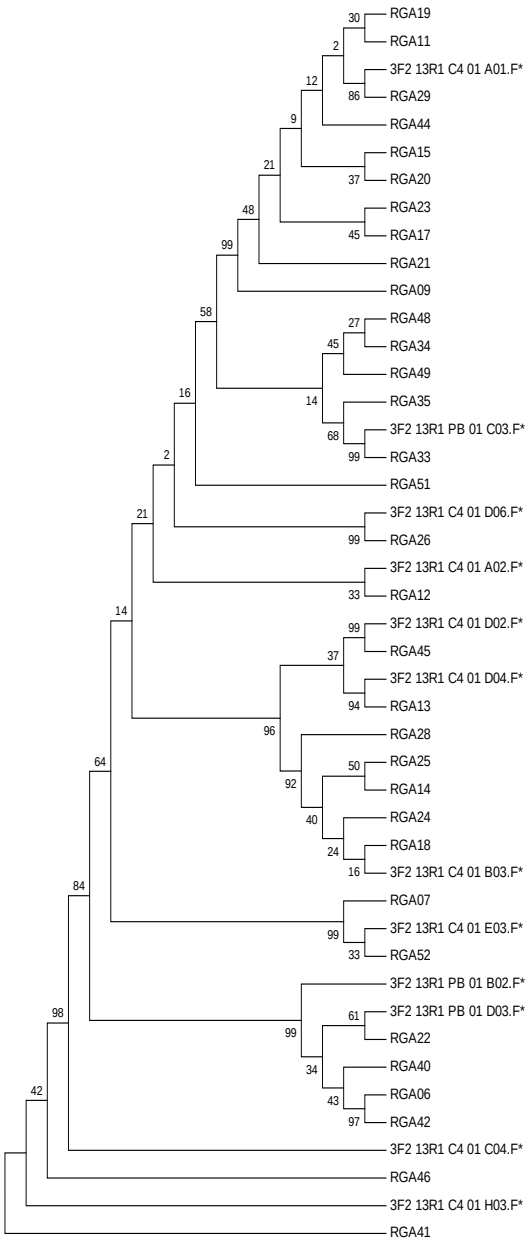

Figure 1 Phylogenetic analysis of common aligned amino acid sequences of 45 Musa contig sequences. (* sequences amplified in the current study)

Analysis of assembled RGA sequences

Assembly of the sequences amplified from *M. acuminata* Calcutta 4 and Pisang Berlin generated a total of 18 contigs and singletons with homology to RGAs (46%). Many showed uninterrupted open reading frames (ORFs) encoding RGAs, with the remainder containing premature stop codons, and/or frameshifts. These latter sequences are likely derived from pseudogenes, PCR mutants or artefacts. Analysis of contigs and singletons with homology to R-genes or RGAs revealed nine specific to Calcutta 4, and four to Pisang Berlin. Five contigs contained sequences from both cultivars.

Phylogenetic analysis

A phylogenetic analysis of common aligned amino acid sequences was conducted on 45 *Musa* contig sequences, which included 33 RGA contig sequences previously amplified in Calcutta 4 using eight NBS-LRR-targeting primer combinations [4] (Figure 1). *Musa* sequences were

divergent, indicating the presence of a diverse gene family coding for proteins with NBS-LRR domains. Although potentially dependent upon sample size, clades did not appear to be cultivar specific.

Continued work is underway with the primers targeting additional R-gene families, and on further cultivars contrasting in resistance to BLSD. *Musa* RGAs have previously been used by our group for selection of clones from *M. acuminata* Calcutta 4 (AA), *M. acuminata* Grande Naine (AAA), and *M. balbisiana* Pisang Klutuk Wulung (PKW) (BB) BAC libraries [4]. Such studies are also planned with the additional RGAs characterized in this study. Full length sequencing of BAC clones offers potential for identification of complete R-gene nucleotide sequences, applicable both in transformation and breeding programs for banana genetic improvement.

ACKNOWLEDGEMENTS

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Enhancing Drought and Salinity Tolerance in Wheat Crop Grown in the Mediterranean Region

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Abstract

Drought and salinity are major constraints on crop production and food security, and adversely affect entire countries over several years resulting in serious social, economic, and environmental costs. Water is in an extremely short supply in up to 10 eastern and southern Mediterranean countries. Wheat production in the Mediterranean region is limited mainly by the availability of water resources. Investigating the mechanisms by which wheat physiologically adapts to water deficits points to a salinity tolerance strategy showed that varieties of wheat which are able to maintain photosynthesis and growth at low soil Ψw often display a relatively greater capacity for leaf osmotic adjustment. Understanding the molecular basis of salt-stress signaling and tolerance mechanisms in wheat is required for engineering local wheat genotypes more tolerant to salt stress. This goal can be achieved only by first deciphering the physiological responses of wheat to salt stress. Recent work at the molecular level has led to the identification and cloning of cDNAs encoding proteins which are involved in the cellular-level physiological system which facilitates this adaptive response. Transgenic *Arabidopsis* plants over-expressing wheat candidate genes encoding ion transport proteins (TNHX1, SOS1, TVP1), or dehydrin (DHN-5) are much more resistant to high concentrations of NaCl and to water deprivation than the wild-type strains. Over-expression of the isolated genes from wheat in *Arabidopsis thaliana* plants is worthwhile to elucidate the contribution of these proteins in the tolerance mechanism to salt and drought. Testing candidate genes in TILLING available wheat population will allow the identification of new alleles conferring abiotic stress tolerance.

Introduction

Wheat production in the Mediterranean region is limited mainly by the availability of water resources and soil salinity. This problem is more acute when irrigation procedures use poor quality water and when soil drainage is poor. This has led to serious loss of yields in many arid and semi-arid regions in the World. Plants have developed different strategies to face water deficit and over the past few years, much attention has been focused on the identification of genes and proteins induced in response to environmental stress [1, 2]. Developing embryos have been largely used as an experimental model to study desiccation tolerance, due to their ability to survive extreme water loss during the final maturation stage of development. Some of the polypeptides accumulating in the mature embryos are directly involved in dehydration tolerance and in many cases, similar polypeptides have been shown to accumulate both in dry embryos and in vegetative tissues of plants subjected to stresses such as dehydration, cold, high salinity and abscisic acid (ABA) treatments [3, 4].

In wheat, one of the major mechanisms conferring salt tolerance is sodium exclusion from the leaves [5, 6, 7]. Sodium exclusion is achieved by means of low net uptake by cells in the root cortex and tight control of net unloading of the xylem by parenchyma cells in the stele [8, 9]. The enhanced K^+/Na^+ discrimination trait in bread wheat (*Triticum aestivum*, AABBDD) cultivars confers some degree of salt tolerance [10]. A locus for the trait, *kna*1, was mapped to the distal region of chromosome 4DL [11]. In contrast, tetraploid durum wheat (*Triticum turgidum* L. subsp. *Durum*, AABB) cultivars which are more salt-sensitive than bread wheat [12, 13], lack this trait. A homologue of the *kna*1 locus has not yet been found on either the A or B genomes of tetraploid wheat. Genetic analysis of Na^+ exclusion in *durum* wheat showed that, in a low leaf blade Na^+ phenotype, Na^+ exclusion was controlled by two dominant loci that are interactive (epistatic) rather than additive [14]. These loci were designated *Nax1* and *Nax2* (Na^+ exclusion loci). Recently, a molecular marker linked to *Nax1* was identified and has facilitated the rapid transfer of this trait into commercial varieties of *durum* wheat [15]. Physiological studies indicated that in a tolerant genotype, the control leaf blade Na^+ concentration is the result of the interaction between net xylem loading and leaf sheath sequestration [16]. Species that cannot exclude salt from the transpiration stream must also be able to compartmentalize the salt in vacuoles, thereby protecting the cytoplasm from ion toxicity and avoiding build-up of rigid cell wall, which would cause dehydration [17]. If Na^+ is sequestered in the vacuole of the cell, K^+ and organic solutes should accumulate in the cytoplasm and organelles to balance the osmotic pressure of the Na^+ in the vacuole. Na^+ sequestration into the vacuole depends on expression and activity of Na^+/H^+ antiporters as well as on V-type H^+-ATPases and H^+-PPases. These phosphatases generate the necessary proton gradient required for activity of Na^+/H^+ antiporters and homeostasis equilibrium. Furthermore, sodium efflux from root cells prevents accumulation of toxic levels of Na^+ in the cytosol and transport of Na^+ to the shoot. Molecular genetic analyses in *Arabidopsis* sos mutants have led to the identification of a plasma membranes Na^+/H^+ antiporter, SOS1, which plays a crucial role in sodium efflux from root epidermal cells and the long distance Na^+ transport from root to shoot under salinity [18]. Studies of the physiological response of wheat to drought and salt stress are very important to understand the molecular bases on gene expression. The aim of this study was to determine the physiological correlates of the genetic control of leaf Na^+ in durum wheat to facilitate screening for novel traits and to enhance understanding the mechanism of saline tolerance in the two wheat genotypes. Expression patterns of candidate genes, *TmHKT1; 4-A2*, *TNHX1* and *SOS1* was studied in roots, sheaths and leaves of the two wheat genotypes to determine traits for improving control of Na^+ uptake and transport. As salinity stress negatively affects survival, growth, and development of crop plants, owing to irrigation practices and increasing demands on fresh water supply, engineering of salt-tolerant crop plants could provide an acceptable solution to the reclamation of farmlands lost to agriculture because of salinity and lack of rainfall. In this study, it is shown that transgenic *Arabidopsis thaliana* plants over-expressing one of the two wheat cDNAs encoding the tonoplast H+-PPase (TVP1) or the Na+/H+ antiporter (TNHX1) are much more resistant to high concentrations of NaCl and to water deprivation than the isogenic wild-type strains. These

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transgenic plants accumulate more Na+ and K+ in their leaf tissue than the wild type.

Materials and Methods

Plant material and stress conditions

Two Tunisian cultivars of Durum wheat *(Triticum durum Desf.),* Mahmoudi (salt-sensitive) and Om Rabia3 (salt-tolerant), were selected for their behavior during saline conditions and used throughout this study. Seedlings from each line were cultured in plastic pots and watered with half-strength modified Hoagland solution by hydroponics system. Plants were grown until the third leaf stage and then we applied salt stress. NaCl doses (0, 50, 100, and 200mM) were progressively applied (salt treatment was adjusted with increasing NaCl concentrations by 50mM per day).

RNA extraction and RT-PCR

Total RNA was extracted from 200 mg of young leaves of Arabidopsis transgenic lines using the RNeasy total RNA isolation kit (Qiagen). To remove contaminating DNA, RNA was treated with RNase-free DNase (Promega). DNase-treated RNAsamples were reverse transcribed using MMLV reverse transcriptase (Invitogen).

Generation of transgenic Arabidopsis plants

The full length TNHX1, TVP1, and Dhn-5 open reading frames (ORFs) were amplified with Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) using wheat cDNA library as template and primers corresponding to the 5' and 3' ends of the wheat candidate genes. The TNHX1 and TVP1 ORF were cloned separately into the BamHI and EcoRI sites of the pCB302.2 plasmid [20]. This binary vector contains a tandem repeat of the cauliflower mosaic virus (CaMV) 35S promoter, the 35S terminator, and the BAR gene for resistance to the herbicide Basta as a selectable marker, between the NOS promoter and terminator. The Dhn-5 ORF was cloned into the NcoI site of the pCAMBIA1302 plasmid resulting in a fusion with GFP (DHN-5::GFP). This vector contains the hygromycine resistance gene (HPT) as a selectable marker, between the 35S promoter and terminator. *Agrobacterium*-mediated transformation was performed via the floral dipping technique of *Arabidopsis thaliana* (ecotype Columbia) [21].

Results

Ion status

Measurement of Na^+ accumulation over 21 days indicated two major differences in Na^+ transport between Om Rabia3 and Mahmoudi. Om Rabia3 genotype sequestered more Na^+ in leaf sheaths than salt sensitive Mahmoudi did when grown in 100 mM NaCl. In contrast, in the leaf blades, Mahmoudi accumulated more Na^+ than Om Rabia3, while the roots of both genotypes accumulated Na^+ to similar concentration (**Fig. 1**). Storage of Na^+ in the leaf sheaths was investigated further by measuring Na^+ content of the leaf sheath and leaf blade after one week of exposure to different levels of NaCl. Although the two genotypes accumulated Na^+ to different concentrations in the leaf sheath, salt tolerant Om Rabia3 accumulated a substantially higher Na^+ concentration than Mahmoudi with no evidence of saturation of storage. The two genotypes seem to have a contrasting capacity to store Na^+ in the leaf sheath and their leaf sheath cells may differ in their ability to extract Na^+ from the xylem stream. This possibility was supported by genotypic differences in the proportion of total leaf Na^+ content that was stored in the leaf sheath. Om Rabia3 sequestered up to 70 % of total leaf Na^+ in the leaf sheath, and this capacity appeared to reach saturation since a slight decrease was observed at 200mM NaCl. In contrast, salt sensitive Mahmoudi stored only up to 40% of leaf Na^+ in the sheath, with little change in response to external NaCl levels. To investigate whether the preferential accumulation of Na^+ in leaf sheaths in Om Rabia3 represented a general solute accumulation mechanism, we measured K^+ in the leaf blade and leaf sheath of both genotypes during 21 d growth in 100mM NaCl. K^+ was accumulated to similar levels in the leaf sheath of each cultivar, whereas in leaf blades, more K^+ accumulated in Om Rabia3 than Mahmoudi giving a higher K^+/Na^+ ratio in the salt tolerant cultivar.

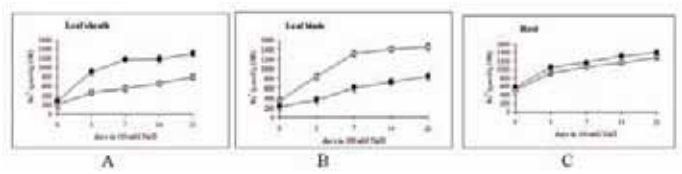

Figure 1 Increase in Na^+ concentrations in the leaf sheaths **(A)**, leaf blades **(B)** and roots **(C)**, of salt tolerant Om Rabia3 (closed symbols) and salt sensitive Mahmoudi (open symbols) during 21 d growth in 100mM NaCl.

Sodium uptake and transport in the plant

Many genes are important in maintaining K^+ or Na^+ homeostasis in higher plants, and could be considered candidates for genetic manipulation. We have studied the expression levels of three candidate genes implicated in improving the control of Na^+ uptake, transport and sequestration: *TmHKT1;4-A2* (*Triticum monococcum* putative sodium transporter; accession number: EF062819 [22]); *TNHX1* (*Triticum aestivum* Na^+/H^+ antiporter; Accession Number: AY296910 [23]); *SOS1* (*Triticum aestivum* plasma membrane Na^+/H^+ antiporter; Accession Number: AY326952 [24]) in *T. monococcum* and the two Tunisian durum wheat varieties: Mahmoudi and Om Rabia3 exposed to 100mM NaCl during two days (**Fig. 2**).

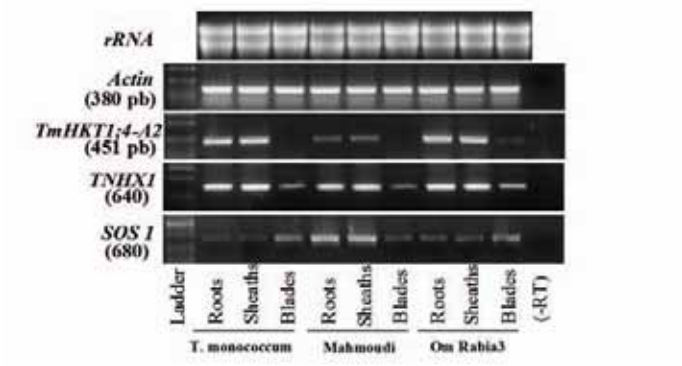

Figure 2 Expression levels of *TmHKT1;4-A2, TNHX-1* and SOS-1 in roots, sheaths and blades of *T. monococcum, T.durum* CVs Mahmoudi and Om Rabia3 using specific gene primers.

Using gene specific primers for RT-PCR analysis, *TmHKT1; 4-A2* was detected highly in roots and leaf sheath of *T. monococcum* and Om Rabia3 and slightly in Mahmoudi, whereas, it was not expressed in leaf blades, consistent with the physiological role of *Nax1* in reducing the Na^+ concentration in blades by retaining Na^+ in the sheaths [25]. The expression levels of TNHX-1 were almost the same in the root and leaf sheath of the three genotypes, however in the leaf blade, there was a high accumulation of TNHX-1 in Om Rabia3 compared to *T.monococcum* and Mahmoudi. For SOS1, which is a plasma membrane Na^+/H^+ antiporter, there is a high accumulation of transcript in the root and leaf sheath of Mahmoudi compared to *T.monococcum* and Om Rabia3. SOS1 and *TmHKT1; 4-A2* could be expressed in an opposite manner when plants are subjected to salt stress.

Molecular characterization of transgenic *Arabidopsis* lines over-expressing the wheat candidate genes (*Dhn-5, TNHX1, and TVP1*)

All transgenic *Arabidopsis* lines expressed the Dhn-5, TNHX1 and TVP1 genes (**Fig. 3A**). Southern blot of genomic DNA of the transgenic lines shows different integration patterns confirming that all lines were derived from independent transformation events (**Fig. 3B**).

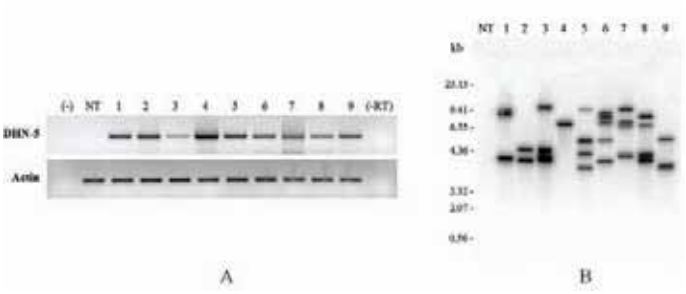

Figure 3 Analysis of the transgenic Arabidopsis lines. **(A)** Analysis by RT-PCR of Dhn-5 expression in *Arabidopsis* transgenic lines. **(B)** Southern blot analysis of genomic DNA of R1 transformants.

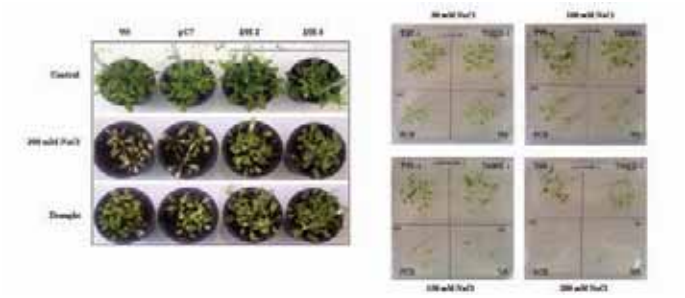

Figure 4 Effect of stress conditions on *in vivo* and *in vitro* cultured *TNHX1, TVP1, and Dhn-5* transgenic Arabidopsis plants. (A) Effect of stress conditions in soil cultured Dhn-5 transgenic Arabidopsis plants. The photograph corresponds to plants at day 10 of water deficit stress and to plants after three days of continuous salt treatment (200mM NaCl). (B) Arabidopsis plants grown in standard culture medium during one week (the first two true leaves have already emerged) were transferred to culture plates containing 50mM, 100mM, 150mM or 200mM NaCl. The photograph was taken 10 days after transplantation, when the transgenic salt-stressed seedlings showed a higher survival rate compared to control.

Increased tolerance of stress treatments of transgenic plants

The observed improvement of tolerance to mannitol in the transgenic Dhn-5 lines is simply displayed by a better capacity to resume growth after osmotic stress caused by the mannitol treatment. When plants grown on soil were subjected to salt stress (200mM) and drought (10 days of water deprivation), a clear difference was also observed between transgenic and control plants. After 10 days of stress treatments (salt or drought), the DH-2 and DH-4 lines continued to grow, albeit at a slower rate, whereas control plants exhibited chlorosis and died (**Fig. 4A**). The homozygous transgenic lines TNHX1 and TVP1 were tested for salt- and drought-stress tolerance. Ten days after germination, Arabidopsis seedlings were transferred to MS medium containing 50–200mM NaCl, and plant survival was monitored. The transgenic plants over-expressing TNHX1 and TVP1 are much more salt tolerant than wild-type or transgenic plants transformed with empty plasmid (Fig.4B). Plants from TNHX1 and TVP1 transgenic lines continue to grow well in the presence of 100–200mM NaCl, whereas wild-type plants and the empty plasmid transgenic line (pCB) exhibit chlorosis and die after 10 days of salt-stress treatment. The toxic effects of 150mM NaCl, with inhibition of growth and development of chlorotic leaves after 10 days in control plants, were delayed and attenuated in the transgenic plants. In plants grown on soil under salt stress (200mM NaCl) or drought (10 days of water deprivation), controls showed growth reduction and exhibited chlorosis, whereas the transgenic lines survived and continued normal growth.

Discussion

Over-expression of the wheat vacuolar Na^+/H^+ antiporter *TNHX1* and H^+-pyrophosphatase *TVP1* in transgenic *Arabidopsis* plants results in salt and drought tolerance. Wild-type plants displayed progressive chlorosis, reduced leaf area, and a general growth inhibition when treated with high salt concentrations or when deprived from watering. The transgenic plants accumulate more Na^+ and K^+ in their leaf tissue. The increased accumulation of Na^+ and K^+ is likely to be a consequence of the activity of the vacuolar Na^+/H^+ antiporter. We speculate that the antiporter facilitates K^+ as well as Na^+ uptake into vacuoles in exchange for H^+ into the cytoplasm. This compartmentalization may prevent Na^+ toxicity and facilitate cellular K^+ uptake [26]. The Na^+/H^+ antiporter of *Arabidopsis thaliana* (AtNHX1) was shown to mediate the transport of K^+ as well as Na^+ in tomato tonoplast vesicles [27]. The capacity of AtNHX1 to mediate both K^+/H^+ and Na^+/H^+ transport was also demonstrated on reconstituted liposomes with purified AtNHX1 [28]. Moreover, vacuoles isolated from leaves of the *Arabidopsis nhx*1 mutant plants had much lower Na^+/H^+ and K^+/H^+ exchange activity [29]. Alternatively, the increase in K^+ content of leaves in the transgenic lines (TNHX1 and TVP1) may also result from increased Na^+ compartmentalization in leaf cells leading to increased transpiration rates and therefore increased delivery of K^+ to the leaves [30]. The over-expression of the wheat Na^+/H^+ antiporter and H^+-PPase genes in crop plants, including wheat, may be one strategy to engineer agriculturally important plants to withstand these important abiotic stresses. The over-expression of DHN-5 in Arabidopsis has also resulted in an improved tolerance to water stress [31]. However, the resistance to high concentrations of LiCl was not affected in these lines. This finding suggests that the observed tolerance due to DHN-5 depends on an osmotic rather than an ionic tolerance mechanism. This seems to be confirmed by a higher accumulation in the transgenic lines of proline, an osmolyte that is known to contribute to osmotic adjustment. The major aim of our research is an immediate production of transgenic local and regional crop varieties of wheat by ectopic expression of the already proven salt and drought tolerance genes (*TNHX1*, *TVP1*, and *DHN5*). The rationale for improving abiotic stress tolerance in the crop cultivars grown in the Mediterranean countries by over-expressing genes that were shown to confer improved salt and drought tolerance in other plants offers a first-rate possibility to transfer these traits into local crop varieties. We already have taken a Tunisian patent on methods of regeneration and genetic transformation of durum wheat genotypes adapted to the Mediterranean conditions [32]. Callus induction and regeneration of whole plants are already optimized for one Tunisian durum wheat variety. The durum wheat varieties indigenous to the Mediterranean countries are relatively high-yielding in good environmental conditions but do not perform well under drought or in saline soils. Selected varieties representative to the Mediterranean countries are being transformed with either biolistic or *Agrobacterium tumefaciens*.

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Cyto-palynological, Biochemical and Molecular Characterization of Original and Induced Mutants of Garden Chrysanthemum

S K Datta[1,*], K Datta[2] & J Chatterjee[3]

Abstract

A large number of new somatic flower color/type mutants have been evolved by induced mutations in different ornamental plants. Few reports are available on the systematic work being done on the comparative analysis of the original and mutant cultivars. This paper reports the result of comparative analysis on cyto-palynological, biochemical and molecular characters of original and mutant cultivars for a better understanding of the exact mechanism involved in the origin and evolution of flower color mutations. Cultivar identification and cultivar relatedness are important issues for horticultural breeders. Proper characterization and identification of new mutant cultivars is extremely important to protect plant breeder's rights for commercial exploitation.

Introduction

A large number of new flower color/type and chlorophyll variegated mutant varieties have been developed worldwide and commercialized [1-4]. Induced mutagenesis in chrysanthemum is the most successful story. Mutants are phenotypically different from parental variety only in flower color/shape and leaf variegation. There are different opinions regarding the origin of these somatic mutations. Chromosomal aberrations, changes in chromosome number, gene mutation, rearrangement of different histogenic layers and mutation occurring in the biochemical pathway leading to pigment formation are the probable explanations for radiation induced flower color changes. Based on these interpretations, the exact mechanism of induction of somatic flower color mutations cannot be explained with certainty. It is often difficult to distinguish the mutants having a common progenitor or between progenitors. This paper reports results of comparative cyto-palynological, anatomical, biochemical and DNA-based fingerprinting of original and mutant cultivars for a better clear understanding of the exact mechanism(s) involved in the origin and evolution of somatic flower color mutation.

Materials and Methods

Plant materials

Original and Gamma-ray induced mutant cultivars of garden chrysanthemum (*Chrysanthemum morifolium* Ramat.), developed at National Botanical Research Institute, Lucknow, India, were selected as materials for the present analysis.

Cytological preparations

Root tips were collected and temporary squash preparations were made for cytological studies (chromosome counting, chromosomal behavior, karyotype) after staining in Feulgen following the usual hydrolysis procedure [5].

Micromorphology

Flower petals were fixed and then mounted on SEM stubs using double-sided adhesive tape after critical point drying. Subsequently, the materials were sputter coated with gold (200 A thickness) and scanning photomicrographs were taken in JEOL-JSM 35C Scanning Electron Microscope at 10 kV [6].

Pollen morphology

Pollen grains were collected soon after anthesis and pollen slides were made for the study of pollen sterility, size and ornamentations both under light microscope and SEM [7].

Phenolic compounds

Florets were extracted in methanol containing 1% HCl. Chromatograms were developed on glass plates coated with silica gel emulsion. The plates were run in suitable solvent and Rf values of each spot were calculated [8].

DNA extraction and PCR analysis for RAPD analysis

Genomic DNA was extracted from very young leaves by using the DNA extraction procedure in [9]. A total of 40 random primers (Bangalore Genei, India) were tested. Similarity coefficient was calculated [10]. A dendrogram of hierarchial classification was made using the software SPSS 10.0. Pair-wise comparison was done for all genotypes using unweighed pair-group cluster analysis method using arithmetic means [11].

Results and Discussion

Cytological studies

The somatic chromosome number in all the mutant and original cultivars was found to be the same i.e. 2n=(6x)=54, except in parent cv. 'Ratna' and its mutant 'Sonali,' where the chromosome number was 54+2. Few cells with chromosomal aberrations like bridge (**Fig. 1**), fragment, laggard, early separation, clumping, exclusion, micronucleus, etc., were detected during root tip mitosis. Percentage of cells with such aberrations varied from 03 – 2.4%. No mutant specific abnormality could be detected.

The chromosomes of all the materials were characterized on the basis of centromere location and nature and position of secondary constric-

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tion. Comparison of ideograms showed that the mutants did not differ from their respective original cultivars in number of types of chromosomes and number of each type represented in them. The karyotype in the analyzed original and mutant cultivars was reasonably symmetrical [5].

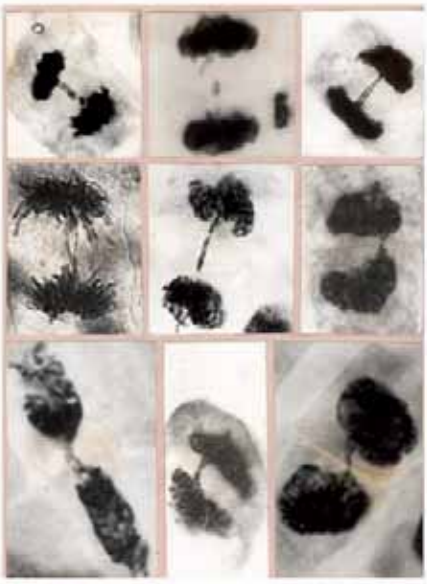

Figure 1 Chromosomal aberrations during root tip mitosis : bridges.

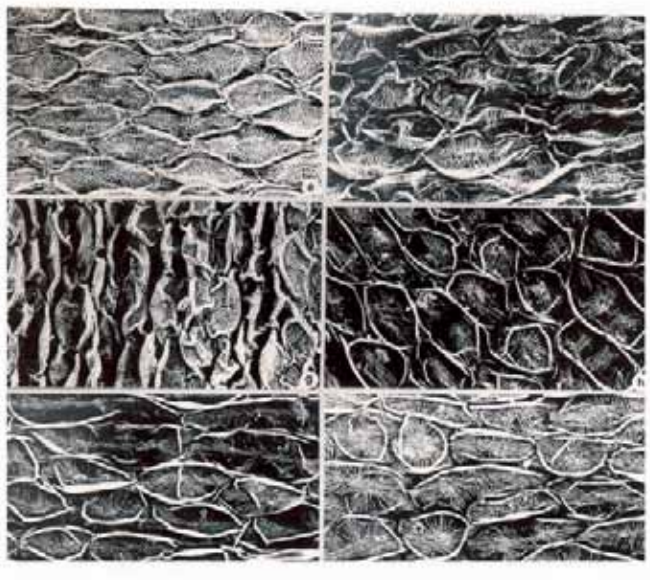

Figure 2 SEM photographs of petal surface of original and mutants of chrysanthemum.

Micromorphological studies

The study on petal epidermal micromorphology reveals considerable variation, particularly in cell boundaries, cell surface, striations and papillae between the original and their respective induced mutant cultivars. It indicated that flower color changes due to mutation were also associated with some changed micromorphology of petal surface (**Fig. 2**). This study clearly indicates that the petal micromorphological characters can be utilized not only for identifying mutants, but also that a correlation study will help the proper identification of chrysanthemum and their origin [6].

Palynological studies

Pollen grains of most of the chrysanthemum cultivars were regular in size, but few cultivars and their mutants had dimorphic pollen grains (**Fig. 3**). The shape of endocolpium was variable; lalongate type was most common but lolongate, circular, square, and indiscernible types were also found. Variation in the shape of endocolpium in the pollen mass was also encountered. Conspicuous changes in exine surface pattern have been observed. The shape of exine spines was variable with regard to base and tip. The exine surface of the original cv. 'D-5' having a fosso-reticulate pattern with narrow muri and irregularly shaped lumina, changed to reticulate exine with broad muri and uniformly circular lumina in the mutants 'Alankar' and 'Agnisikha.' The tips of the spines also changed from straight to bent. The reticulate undulated exine surface of the original cv. 'Kingsform Smith' changed to a scrobiculate wrinkled surface in the mutant cv. 'Rohit.' The punctata exine surface pattern of the original cv. 'Himani' transformed to a scrobiculate pattern in the mutant cv. 'Sheela' [7].

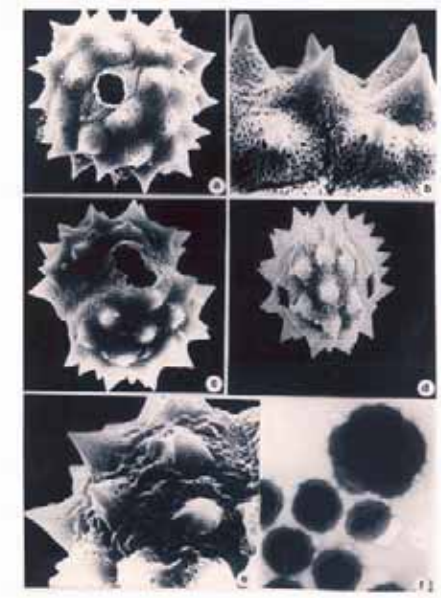

Figure 3 SEM photographs of pollen grains of original and mutant cvs. of chrysanthemum.

Pigment analysis

Pigments of florets/petals of large number of chrysanthemum and rose mutants and original cultivars have been studied by thin layer chromatographic and spectrophotometric methods (**Fig. 4**). Such analyses indicated that somatic flower color changes were due to both qualitative and/or quantitative changes in pigments as a result of mutation/s induced by Gamma-rays in pigment biosynthesis pathway. A schematic representation has been suggested, which explains the probable manner in which differences in chromatographic patter of pigments of original and mutant cultivars may arise [2, 8, 12-14].

RAPD analysis

Out of 40 primers used for RAPD analysis, 10 gave better profiles. Some yielded extremely different banding patterns in mutants and parents.

Bands generated by RAPD fragments were of low molecular weight ranging from 400 bp to 1,500 bp. Bands for each primer ranged from three to 13. This showed a monomorphic banding pattern in all the materials except 'Manbhawan,' where it showed one additional polymorphic band of 550 bp, which made it identifiable from its mother plant 'Flirt' and also from 'Batik,' another mutant of 'Flirt.' Only two primers, i.e. P2 and P3, were able to distinguish some of the mutants from their parents. The similarity among the cultivars and mutants varied from 0.17 to 90% (**Fig. 5**). A low genetic similarity among the mutants and their parents was quite acceptable, as the mutants did not vary greatly in their morphology. A large similarity index would indicate genetically similar backgrounds, while a small similarity index would indicate distantly related backgrounds, and this may explain the wide range of indices (0.17-0.90) found between mutant cultivars and their parents. Although they differed in flower color, bands specific for color could not be distinguished, due to the resolution capacity of tested primers. It is however possible that some of the specific bands present for some of the mutants may code for flower color, but this can only be verified by using SCAR markers and cloning cDN [15].

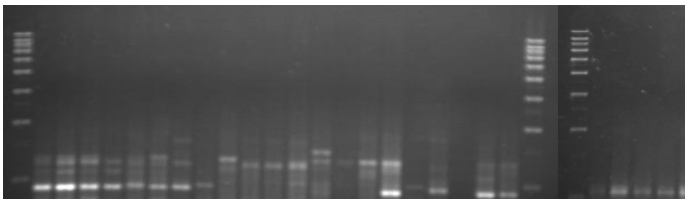

Figure 4 Chromatogram of original chrysanthemum cv. 'E-13' (extreme left) and its five mutants.

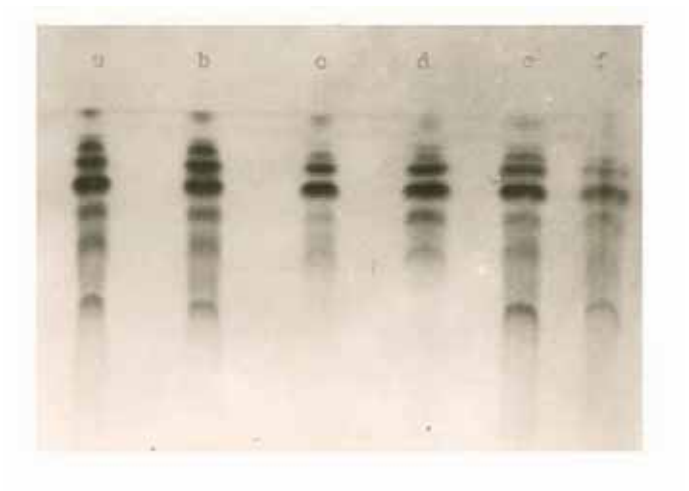

Figure 5 RAPD profile of original and mutant cvs. of chrysanthemum.

Cytological analyses clearly indicated that changes in flower color may be considered to have taken place through gene mutation, but neither through change in chromosome number and aberration nor due to change in karyomorphology. Pollen grain analysis indicated that no appreciable change in pollen apertural character took place in the mutants. Significant changes in pollen exine surface pattern occurred in some of the mutants. These changes were inconsistent and not dose (Gamma-ray) specific. It may be concluded that the changes due to mutation were perhaps induced at several independent loci controlling different characters and that the loci are differentially sensitive to mutagen treatment. Mutants with different flower color could be identified at the molecular level using RAPD technique holding promise to identify unique genes as SCAR markers. A high genetic distance among the different chrysanthemum showed that a possibility exists of introgressing new and novel genes from the chrysanthemum gene pool [15].

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Analysis of EMS Mutagenized Soybean by Combination of DOP-PCR and GS-FLX

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Abstract

Ethylmethanesulfonate (EMS) has been commonly used to induce mutations for various organisms because it led to irreversible mutations with a high level of frequency. With relatively few individuals, saturated mutagenized populations could be generated by chemical mutagens. Since high-throughput sequencing instruments, such as GS-20 or GS-FLX from Roche/454 Life Sciences are now available, characterization of nucleic acids and massive mutant analysis are more feasible. Due to the requirement of sequence information and high cost for designing primers, degenerate oligonucleotide primed PCR (DOP-PCR) instead of direct sequencing was used for single nucleotide polymorphisms (SNPs) survey. In this study, we screen point mutation in soybean mutants generated by EMS mutagenesis using a combination of DOP-PCR methodology and GS-FLX. Four different modified DOP-PCR primers were used for amplifying genomic DNA of three soybean genotypes, Sinpaldalkong 2, SS2-2 and 25-1-1. Then, nucleotide sequences of these amplified PCR products were analyzed by GS-FLX. Different number and length of contigs and singlets were constructed depending on soybean genotypes and nucleotide identity, after sequences were trimmed and aligned. With 100% in identity, average 1,100 contigs and 7,000 singlets were formed in each soybean genotype. In order to survey sequence polymorphisms, POLYBAYES was used with base quality consideration. A total of 1,187 putative SNPs were detected, and these polymorphisms should be reconfirmed by direct sequencing after a homology search against GenBank databases.

Introduction

Inducing mutations is a major factor for studying genetics [1], and using chemical mutagens is the most common and popular method to induce mutations [2]. Alkylating agents, such as EMS, were commonly used to induce mutations for various organisms by formation of adducts with nucleotides, causing nucleotides to miss-pair with their complementary bases [3-7]. Additionally, only low levels of chromosome breaks resulted from EMS mutagenesis. Thus, chemical mutagenesis has become the best method for inducing mutations in genetic studies [2].

Chemical mutagens make it difficult to position mutated sites. Instead of using chemical mutagens, different transgenic technologies, such as tagging or precise targeting, were developed and commonly used in reverse genetics [2]. Simple PCR-based detection for screening mutations is one of the advantages for inducing mutations by new transgenic technologies because changes of amplified fragment size after mutagenesis can be easily identified [4]. But chemical mutagenesis became a popular method for inducing mutations after SNP detection technologies [8] for reverse-genetic mutational screening were rapidly developed.

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Saturated mutagenized populations could be generated by chemical mutagens with relatively few individuals [4]. Also, these chemical mutagens are used in reverse genetics like TILLING (**T**argeting **I**nduced **L**ocal **L**esion **in** **G**enomics) [9-11]. High-throughput technologies were developed for detection of single nucleotide polymorphisms (SNPs) because large numbers of mutated individuals were generated in TILLING [4]. Direct sequencing combined automated polymorphism discovery programs is the original method for SNP discovery [11-14]. Denaturing high-pressure liquid chromatography (DHPLC) [15-17] and CEL1 endonuclease cleavage followed by polyacrylamide gel electrophoresis analysis [2, 18] are also commonly used for SNP survey as the non-sequencing strategies.

Due to the requirement of sequence information and high cost for designing primers, DOP-PCR instead of direct sequencing was used for SNPs survey [19]. Since high-throughput sequencing instruments, such as GS-20 or GS-FLX from Roche/454 Life Sciences are now available, characterization of nucleic acids and massive mutant analysis are more feasible [20-23].

In this study, we screen point mutation in soybean mutants generated by EMS mutagenesis using a combination of DOP-PCR methodology and GS-FLX.

Materials and Methods

Four different modified DOP-PCR primers were used for amplifying genomic DNA of three soybean genotypes, Sinpaldalkong 2 (recommended cultivar in Korea) [24], SS2-2 (EMS induced mutant from Sinpaldalkong 2 [25] and 25-1-1 (M_3 regenerated plant from EMS-treated immature embryo cultures of Sinpaldalkong 2). Genomic DNA from three soybean genotypes were isolated by [13] and DOP-PCR was performed as described by [19]. Then nucleotide sequences of these amplified PCR products were analyzed by GS-FLX.

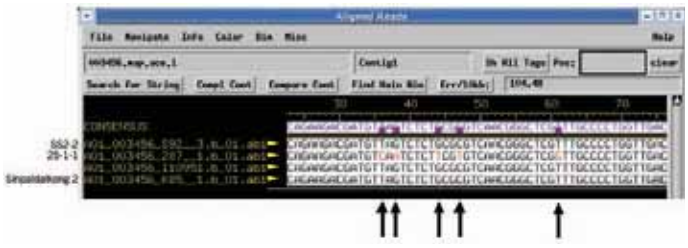

Figure 1 A portion of aligned GS-FLX sequences of soybean genotypes. Putative SNPs are indicated by arrows.

Results and Discussion

Modified DOP-PCR primers were trimmed by custom PERL scripts and the TGICL tool was used for alignment and assembly [26]. Different number and length of contigs and singlets were constructed depending on soybean genotypes and nucleotide identity (98% or 100%), after

sequences were trimmed and aligned (**Table 1**). At 98% in identity, more than 1,400 contigs and 4,300 singlets were formed in each soybean genotype. The numbers of contigs and singlets were increased in 100% of identity; average 1,100 contigs and 7,000 singlets (**Table 1**).

In order to survey sequence polymorphisms, only orthologues showing 100% of identity were used to avoid possible inclusion of paralogue sequences. A total of 1,187 putative SNPs were identified by POLYBAYES with base quality consideration (**Fig. 1**). These polymorphisms will be further analyzed by direct sequencing or DHPLC after homology searches are performed against GenBank databases.

Table 1. Numbers of contigs and singlets and their lengths after sequence alignments by nucleotide identities

Soybean genotypes	Identity	Contigs		Singlets		Total	
		Number	Length (bp)	Number	Length (bp)	Number	Length (bp)
Sinpaldalkong 2	98%	1,649	356,933	5,101	695,133	6,750	1,052,066
	100%	1,150	249,989	7,722	1,144,077	8,872	1,394,066
SS2-2	98%	1,460	235,296	5,211	520,144	6,671	755,440
	100%	1,130	176,262	7,085	775,004	8,215	951,266
25-1-1	98%	1,433	304,703	4,387	561,251	5,830	865,954
	100%	1,038	215,282	6,447	910,336	7,485	1,125,618

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Improvement of Mutant Wheat for Baking Quality Using Marker-assisted Selection

B Naserian Khiabani*, M J Zamani, M T Hallajian & C Vedadi

Abstract

Cultivars carrying the alleles HMW*x*2 and HMW*x*5 were clasified according to results of SDS-PAGE in polyacrylamide gel. Based on SDS-PAGE results we made crosses between Bezostaya, Enia, Tajan, Chamran, Pishtaz as pollinators (with high bread making quality) and Tabasi mutant lines (low bread making quality, but with other desirable traits) as recipient parents. F_1 and F_2 plants subsequently obtained from F_1 hybrids were planted in a greenhouse. Selection for high baking quality was performed using an STS marker related to Glu1D (subunits 5+10) that showed a sharp band (450bp) in all genotypes that have 5+10 subunits. The identification of *Glu-D1* HMW*x*5 carrier genotypes is more straightforward at the gene than at the gene product level. Furthermore, in all blind experiments, including a wide array of wheat genotypes, the PCR system correctly detected the presence of the *Glu-D1* HMW*x*5+*y*10 pair. F_2 individuals that had this allele were selected and planted in the field in April 2007. Selection of F_3 individuals was done according to agronomical traits such as earliness, vigor and yield components, such as seed number per spike, weight of seed per spike, etc.

Introduction

Wheat is one of the three most important crops in the world, together with maize and rice. Approximately 600 million tons are harvested annually with cultivation extending over a vast geographical area, including higher elevations in the tropics. Dough of bread wheat (*Triticum aestivum* L., AABBDD, n = 7) has unique properties, the most important of which is the elasticity of its gluten (the product of wheat storage proteins). This special property allows for the baking of bread, which has been a basic food for man throughout recorded history, and probably for a much longer period; it remains the principal food product made from wheat [1]. The physical properties (rheological properties) of wheat-flour doughs include their extensibility and resistance to extension, which in turn have a major influence on their mixing behavior, and are important factors in determining the bread making quality of a wheat variety. Wheat gluten proteins consist of two major fractions: the gliadins and the glutenins [2].

The proteins, called high molecular weight glutenins (HMW-GS), make dough elastic and allow it to trap the gas bubbles produced by yeast and to rise [3]. Moreover, bread-making performance has also been related to the glutenin polymer size distribution. Cultivars of high bread making quality have high proportions of glutenin polymers of high molecular size [4, 5]. Bread-making quality of wheat genotypes is determined by specific alleles of high-molecular weight glutenin subunits (HMW-GS) and can be improved by integration and expression of specific HMW-GS genes. Alleles for HMW glutenins encoded at the Glu-1 loci have been shown to contribute to bread making quality [6, 7, 8, 9]. A strong association with gluten strength was reported in many studies for the 5 + 10 subunit pair encoded at Glu-D1 locus [6, 7]. Different *x* and *y* subunit combinations encoded at the Glu-B1locus have also been reported to have different effects on gluten strength [10].

Although the protein fraction of wheat seed known as gluten is associated with baking quality, High Molecular Weight (HMW) glutenins are the subunits most closely associated with this trait. Bread wheat could in theory, contain six different HMW-GS, but due to the "silencing" of some of these genes, most common wheat cultivars possess three to five HMW-GS. Bread wheat could, in theory, contain six different HMW-GS but due to the "silencing" of some of these genes, these proteins have conserved amino and carboxy domains with Cys residues and a repetitive hydrophobic central domain. The homologous loci *Glu*-A, *Glu*-B and *Glu*-D control the synthesis of HMW glutenins and are found on linkage group 1 of their respective homologous genomes in wheat. Each loci encodes two subunits of different molecular weights, *x* and *y*. These subunits present a tight genetic linkage and are frequently reported as the *x*+*y* pair. Considering HMW-GS composition, the most frequent subunits at Glu-A1 locus were N, at Glu-B1 locus 7+9 and at Glu-D1 locus 2+12. The cultivars with the GS 5+10 at Glu-D1 locus have shown better technological characteristics in contrast to cultivars with the GS 2+12. Several independent studies report the close association between the allelic pair HMW*x*5+*y*10 at the locus *Glu-D1* and improved baking quality, whereas the opposite applies to the allelic pair HMW*x*2+*y*12 [11, 12].

The genetic analysis of wheat is hampered by its large genome size (3,500 Mb, being 1 Mb 1,000,000 pairs of bases) and hexaploid nature (there are three genomes: A, B and D in cultivated wheat). Traditional methods to select wheat segregant lines carrying "good" baking quality alleles are based upon sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) systems or the rheological analysis of advanced breeding lines. However, according to [13] there are known discrepancies between derived molecular weights and their order of migration on SDS-PAGE for some HMW glutenins. Since even medium size wheat breeding programmes handle over 10,000 genotypes per year, steady and cost-effective systems based on the identification of genes rather than gene products are required. Once such systems are developed and verified they can be easily up-scaled and expanded, taking advantage of Polymerase Chain Reaction (PCR) procedures. Molecular markers were used to identify the allele/gene composition of complex loci *Glu-A1*and *Glu-B1* of high-molecular-weight (HMW) glutenin subunits in triticale cultivars by [9, 10, 11, 12]. Schwarz, *et al* [14] showed that the PCR assay can be applied for the detection and negative selection of the 'poor bread-making quality' Glu-B1-1d (B-x6) alleles in wheat breeding programmes. The availability of DNA sequences of a number of HMW glutenin alleles allowed the design of specific PCR primers that could be used to assess the allelic variation at the Glu-1 loci [12].

Bustos, *et al* [15] described the cloning and characterization of the nucleotid sequence belonging to the *x-null* allele of this gene. Their results showed the usefulness of the method in selecting recombinant plants with desired glutenin alleles using molecular markers at any moment in plants life cycle. Accuracy, simplicity and speed make the proposed PCR

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generated DNA markers a valid alternative to standard techniques for selecting genotypes containing the high-molecular-weight glutenin subunits related to bread quality. Moreover, MAS could allow the screening of hundreds of plants in one day for a quick, early-generation evaluation, thereby saving time and resources otherwise required for seed increase and physical quality testing in a wheat breeding programme [10].

Here, we looked for DNA polymorphisms between the HMW glutenin alleles Glu-1D (5+10 and 2+12). Also, we used this polymorphism in detecting high baking quality amongst F_2 and F_3 individuals.

Materials and Methods

Experiments were conducted in the Molecular Marker Lab of the Nuclear Agriculture Department of the Agriculture Medicine and Industry Research School, Iran, with 49 cultivars/mutant lines selected for this project. These genotypes are 34 mutant lines produced by gamma irradiation of some Iranian cultivars (Tabasi, Roshan, Omid, Azar, Azadi) with 150Gy, 200Gy and 250Gy in 1995 by Majd, *et al*, and 15 commercial cultivars. Cultivars carrying the alleles HMW*x*2 and HMW*x*5 were chosen according to results of SDS-PAGE in polyacrylamide gels.

For transferring of 5+10 subunits to mutant lines that did not have good quality, crosses were done between Bezostaya, Enia, Tajan, Chamran, Pishtaz as pollinators (with high bread making quality) and Tabasi mutant lines (low bread making quality) which have desirable traits as recipient parents. F_1 and then F_2 plants derived from F_1 were planted in a greenhouse. Genomic DNA of each individual in the F_2 generation was extracted by mini-CTAB methods from leaves, and was quantified via spectrophotometer measurement of UV absorption at 260 nm and by means of 1.2% agarose gel electrophoresis with ethidium bromide florescence, with a 100 bp DNA ladder (SMO321 Fermentas) as DNA size marker. Selection for high baking quality was performed using STS Marker [16] related to Glu1D (subunites5+10). This primer (5›GCCTAGCAACCTTCACAATC3›, 5› GAAACCTGCTGCGGACAAG 3›) reproduced a single sharp band (450bp) in all genotypes that have 5+10 subunits. Optimized PCR conditions were 50 ng of DNA; 300 mM each dNTP, 3 mM $MgCl_2$; 10 pmol each primer and 0.6 U *Taq* DNA polymerase (all purchased from MBI Fermentas) in a 25 µl volume. A Tgradient thermocycler (Biometra) was used and the PCR programme included 25 cycles at 94°C for 1 min; 63°C for 45s and 72°C for 30 s and final extension at 72°C for 5 min (annealing temperature was slightly changed according to Primers). PCR products were soaked at 4°C and separated through electrophoresis. Agarose gels (1.5%) were prepared in 1X TAE buffer and electrophoresis was conducted at 100 V. Gels were then stained with Ethidium Bromide, digitized and stored. All the bands were compared with 100bp ladder (SMO321 Fermentas) as DNA size marker.

Results and Discussion

Individuals were selected according to baking quality using STS markers as well as agronomical traits in the F_3 and F_4 generation under field condition.

Table 1 and Fig. 1 show SDS-PAGE results, where none of the Tabasi mutant lines has 5+10 submits. Instead of 5+10, all the Tabasi mutant lines have 2+12 subunits. Only some of the commercial cultivars such as Bezostaya, Enia, Tajan, Chamran, Pishtaz, showed 5+10 subunits. Generally, in Iranian wheat, frequency of 2+12 is more than 5+10; the frequency of 2+12 subunits was just over 75%. In contrast, the frequency of 5+10 that was less than 25 % (**Table 2**). In addition, other subunits like 13+16 and 17+18 in gluB1 locus that had positive effect on baking quality were seen in low frequency, so that most of the mutant genotypes had low quality. On the other hand, a commercial cultivar that was used as control had high baking quality. According to [4] and [5], there is a significant relationship between the presence or the absence of some specific high molecular weight (HMW) glutenin subunits and bread making quality of wheat varieties. The most important subunits are 5+10 and 2+12, which are expressed from the Glu - D1 loci.

Considering HMW-GS composition, the most frequent subunits at Glu-A1 locus were N, at Glu-B1 locus 7+9 and at Glu-D1 locus 2+12. The cultivars with the GS 5+10 at Glu-D1 locus have shown better technological characteristics in contrast to cultivars with the GS 2+12. Several independent studies report the close association between the allelic pair

Table 1. High Molecular Weight (HMW) quality score for some Iranian mutant wheat

NO	Genotype	Glutenin subunit			Score
		1A	1B	1D	
1	ChS	N	7+8	2+12	6
2	Bezostaya	2*	7+9	5+10	9
3	Enia	1	7+8	5+10	10
4	Kraj1	N	7+9	5+10	7
5	Pishtaz	2*	7+8	5+10	10
6	Chamran	2*	7+9	5+10	9
7	Navid	2*	7	5+10	8
8	Atrak	2*	7+9	5+10	9
9	Hot Tajan	2*	17+18	5+10	10
10	Tajan	2*	13+16	5+10	10
11	Tabasi	N	7+8	2+12	6
12	T-65-9-1p	2.1	7+8	2+12	6
13	T-66-58-6	N	7+8	2+12	6
14	T-66-58-9	N	7	2+12	4
15	T-65-6	2.1	7+8	2+12	6
16	T-65-5-1	2.1	7+8	2+12	6
17	T-66-58-10	2.1	7+9	2+12	5
18	T-66-58-12	N	7+9	2+12	5
19	T-65-4	N	7+8	2+12	6
20	T-65-9-1	N	7+9	2+12	5
21	T-67-60	N	7+8	2+12	6
22	T-65-7-1	N	7+8	2+12	6
23	T-66-58-8	2*	13+16	2+12	8
24	T-66-I-II				?
25	T-65-9-II-4	N	17+18,9	2+12	6
26	T-65-58-14	1	6+8	2+12	6
27	T-66-58-7	N	-	2+12	3
28	As-48	2*	6+8	2+12	6
29	Asadi	2*	7+8	2+12	8
30	Azar	2*	7+8	2+12	8
31	Az Mutant	2*	6+8	2+12	6
32	Omid	N	7+8	2+12	6
33	O-64-4	2.1	7+8	2+12	6
34	O-64-1-1	2.1	7+8	2+12	6
35	O-64-1-10	N	6+9	2+12	3
36	Roshan	N	7+8	2+12	6
37	Ro-1	N	7+8	2+12	6
38	Ro-3	N	7+8	2+12	6
39	Ro-6	N	9	2+12	3
40	Ro-9	N	17+18	2+12	6
41	Ro-10	N	6+8	2+12	4
42	Ro-12	N	7+9	2+12	5
43	Ro-11	N	7+8	2+12	6

HMW*x*5+*y*10 at the locus *Glu-D1* and improved baking quality, whereas the opposite applies to the allelic pair HMW*x*2+*y*12 [11, 12].

Table 2. Frequency (%) of the high molecular weight glutenin subunits (HMW-GS) in wheat cultivars

Locus	Bands	Alleles	Cultivars/mutant lines	% of frequency
Glu-A1	2*	b	12	28.57
	N	c	22	52.39
	1	a	2	4.76
	2.1	d	6	14.28
	7	a	2	4.77
	7+8	b	20	47.61
	7+9	c	8	19.04
Glu-B1	6+8	d	4	9.52
	13+16	f	2	4.76
	17+18	l	3	7.14
Glu-D1	2+10	e	33	78.57
	5+10	d	9	21.43

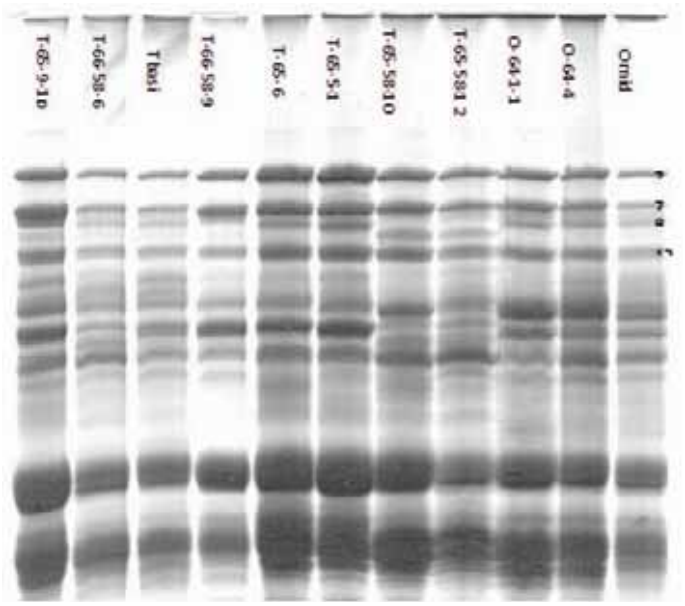

Figure 1 HMW Glutenin electrophoregrams of the wheat (Tabsi mutants and Omid mutants).

P5P6 primer produced a 450 bp (**Fig. 2**) sharp and clear band in genotypes that have DX5 allele. Using this primer, F_2 individuals having 5+10 allele were selected and, in April 2007, were planted in the field, where F3 individuals were selected according to various agronomic traits, i.e. earliness, vigor, etc., and plants with high performance were selected. These plants will be planted in field conditions. Some F_2 plants showed a new band at 1,350 bp. This band was observed in both the genotypes that showed 450 bp band or not, so that it needs to be sequenced to determine its possible role in baking quality and also to define it for new recombinant alleles. According to [17] and [18], there is a clear genetic association between baking quality and HMW glutenins allelic composition in wheat. Since there are only two main reported alleles in the HMW-D locus, detecting the presence or absence of just one allele allows deducing the allelic composition of any given wheat sample. Traditional methods to select wheat segregated lines carrying "good" baking quality alleles are based upon sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) systems or the rheological analysis of advanced breeding lines. The direct use of DNA information is a valuable tool to identify segregating breeding lines useful for plant breeders. The system reported here allows the steady identification of wheat breeding lines carrying HMW*x*5 alleles.

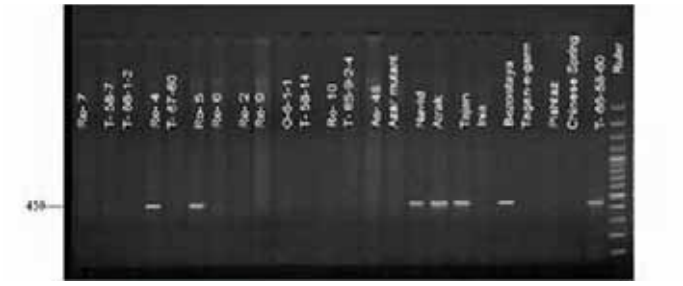

Figure 2 PCR product by P5P6 primer, this primer represent DX5, the genotypes that carrier this subunit produced a 450 band, while other genotypes that have DX2 didn't produce any band.

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NOTES

The year 2008 will mark the 80th anniversary of mutation induction in crop plants. The widespread use of induced mutants has led to date to the official release of close to 3000 mutant plant varieties throughout the world. Many of these varieties have been widely grown by farmers, both in developed and developing countries, resulting in considerable positive economic impacts that are measured in billions of USD. In the past decade, induced mutations have become a means of choice for the discovery of genes that control important traits, and for understanding their functions and mechanisms of actions. The papers included in this book present some of the significant achievements, demonstrate the current development, and outline the perspectives in this dynamic field.

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