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New Intelligent Tools to Understand Seed Development and Possible Implications to Molecular Farming

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Abstract: Understanding the events controlling endosperm development and it's genetic regulation may give new possibilities for molecular farming, functional foods and fish feed. The cereal endosperm represents the worlds major source for food, feed and industrial raw material. When fully developed, the endosperm is a simple plant system consisting of four major cell types, the starchy endosperm, the aleurone layer, the transfer cells, and cells of the embryo surrounding region. Our work in maize, rice and barley, is complemented with studies in *Arabidopsis*, with increasing opportunities with comparative genetics with the genome of Arabidopsis sequenced and rice to come. An understanding of the mechanisms underlying endosperm development in general and cell fate specification in particular, is expected to facilitate alterations in grain quality as well as quantity. Results from reverse approaches to identify regulatory control elements directing preferential endosperm expression in transgenic maize, rice and barley for different promoter regions (*LTP1, LTP2, B22E* and *AGPase*) will be discussed. When genes controlling cell identity are identified through mutant studies, genetic screens and transgene studies underway, this knowledge can be used to generate cereals to meet special needs.

keywords: Cereal Seed Development, DNA Sequence

Introduction

Entering the post-sequencing era with the genomes of increasing numbers of organisms completely sequenced, opens new possibilities using bioinforamtics to understand life. The interest in promoter analysis received a boost with the arrival of microarray gene-expression data. Studying genes with similar expression profiles, i.e. cell specific localization, timing or similar developmental expression, should give us some answers as to which genes that have similar regulation and which have not, and to how similar regulation might be controlled. However, this increasing amount of experimental results demands sophisticated models and analysis to identify the biological significance of i.e. the DNA sequences to genetic regulation and developmental control.

Our research focus is cereal seed development, using both forward and reverse genetics. We are studying mutant phenotypes of barley and maize in order to isolate genes with important functions in seed development (Spillane *et al.* 2001). Additionally, from different genetic screens, we have isolated a number of genes preferentially expressed during seed development and located to specific cells types of the endosperm (Becraft *et al.* 2000) (Opsahl-Ferstad and Rudi 2000). However, identifying these genes functions has proven very difficult. Transformation of cereals still generates low numbers, and obtaining gene 'knock-outs' in plants is still very difficult (Kumar and Matthias 2001). Even though silencing could have been beneficial in creating 'knock-outs' it demands consistent and complete transcriptional or post transcriptional silencing in order to possibly create mutant phenotypes. We still don't have a reliable method of creating such gene 'knock-outs' in seeds (Jones *et al.* 1999). In addition to this we have the problem of redundancy in i.e. maize (allotetraploidization) creating problems in generating mutant phenotypes even from huge mutant collections. The development of homologous recombination during transgene integration in plants is promising and could help ensuring single copy integration to prevent silencing of the transgene, positional effects and be used to obtain gene 'knock-outs' and mutant phenotype (Kumar and Matthias 2001).

Endosperm Development – Cellularization and Formation of Four Cell Types: Following double fertilization, the triploid endosperm nucleus divides without cytokinesis to produce a large peripheral syncytium surrounding the central vacuole. Complete cellularization of the young endosperm is a result of repeated cycles of anticlinal wall growth and periclinal divisions. Each nucleus of the cyncytium gives rise to an outer aleurone and an inner starchy endosperm cell initial respectively (Olsen *et al.* 1998). Early embryo and endosperm development is recently reported to be mainly under maternal control through imprinting of the paternal genome, before the zygotic genes seem to be activated first three to four days after fertilization (Vielle-Calzada J-P 2000). The rate of transcription has previously been found to be six fold increased at the period of barley endosperm cellularization, from 3 to 7 days after pollination (DAP) (Bosnes and Olsen 1992). How the early endosperm development is regulated and controlled through cell divisions, the microtubules, division planes, cell wall formation and signalling is about to be understod (Becraft and Asuncion-Crabb 2000) (Olsen 2001). The two main types of cells specified in the cereal endosperm development are the outer aleurone cells and the inner starchy endosperm cells. The Dek1 and Crinkly4 mutants have patchy or lacking aleurone cell development, implying a role of the corresponding genes in aleurone cell identity and the dek1 genetic signal seem necessary through quite some time of the development in order to keep the aleurone identity and avoid a transition to starchy endosperm appearance (Becraft and Asuncion-Crabb 2000) (Thompson 2000). This indicates that starchy endosperm identity is the default patway, giving the cells starchy endosperm identity unless other signals keep 'ordering' i.e. aleurone identity. *Crinkly4* encodes a putative receptor kinase with a cysteine-rich region in the extracellular domain similar to the ligand binding domain

in mammalian tumor necrosis factor receptors (Becraft *et al.* 1996). When looking at transgenic rice for 5' deletion constructs of aleurone specific promoters driving Gus (unpublished results), one also get the impression that aleurone identity depend on a signal possibly from the surrounding maternal tissue and that a certain gradient might set up aleurone versus starchy endosperm identity (manuscript in prep.). Whether these genes are receptors and the signal is a ligand in one or several alternative putative genetic cascades, will hopefully be answered in the near future. *ZmOCL1* (Ingram *et al.* 1999) is another gene possibly involved in setting up outer cell layer, L1, identity being expressed in the outer cells of the endosperm from very early stages (before 3 DAP) of the syncytium till just after cellularization (5 DAP) where the expression starts to diminish 7 DAP (Ingram *et al.* 1999). *Dek1* is specifically expressed in the endosperm aleurone cells, while *Crinkly4* is also expressed in the epidermis of leaves and *ZmOCL1* is expressed in the presumed L1 layer of all plant parts being very early embryos, early endosperm, primary root and shoot meristems and developing leaves and floral organs (Ingram *et al.* 1999).

The Basal Endosperm Transfer Cells: When doing differential screening for endosperm preferentially expressed genes, the earliest gene reported in barley from our group, *NUC1*, is expressed in the maternal nucellus, antipodal cells and stigmata before fertilization, and at later stages in the nucellar epidermis and nucellar projection (Doan *et al.* 1996). *END1* is specifically expressed in the endosperm coenocyte of barley, in the area over the nucellar projection during initial cellularization and anticlinal cell wall formation (Doan *et al.* 1996). This indicates that a positional signal for transfer cell differentiation is in place already in the syncytium when cellularization and the first anticlinal walls develop, and that this is the first event in endosperm cell differentiation (Olsen *et al.* 1999). These transfer cells, sometimes referred to as modified aleurone cells, are located at the base of the endosperm over the maternal vascular tissue, where they facilitate influx of photosynthates from the green parts of the plant (Weschke *et al.* 2000). The first marker reported for this basal endosperm transfer region, *BET1*, is expressed at later stages in maize (Hueros *et al.* 1995). The *BETL 2-4* are expressed at early and midle stages of endosperm development and encodes predicted small, secreated cystein-rich polypeptides (Hueros *et al.* 1999). The basis for the transfer cell specific gene expression is still unknown.

The Embryo Surrounding Region: One of the earliest genes being activated in the inner maize endosperm, *ESR*, is located in the embryo-surrounding region from 4-5 DAP (Opsahl-Ferstad *et al.* 1997) (Bonello *et al.* 2000). The embryo develops in a pocket within the starchy endosperm, and at early stages, the proximal end of the embryo is physically connected to the endosperm by the suspensor. This part of the endosperm is where internal cellularization is initiated in both cereals and *Arabidopsis italiana* (Bosnes and Olsen 1992) (Boisnard-Lorig *et al.* 2001), possibly explaining why this is one of the earliest preferentially expressed genes to be detected in the inner endosperm. Spontaneous embryo-less mutants do not express the genes, but whether this might indicate that the genes are necessary for the embryo to develop or the embryo is needed for the genes to be activated is still unknow. The *ESR* genes lack introns and encode small polypeptides. Initially no sequence homology could be detected in data base surges. However, CLAVATA3 (CLV3) has given some sequence homology to the ESRs, in a presumed conserved region being affected in two independent mutant alleles of *CLV3*. And recently using more directed tBlastn surge (Zhang and Madden 1997) with additional biological results and sequence information from functional mutant studies, (Cock and McCormick 2001) have just published and interesting overvue of quite a few ESTs and genomic clones having similarities to the putative functional conserved region of ESR2 and CLV3 being named the *CLE* (*CLAVATA3/ESR-related*) genes. Even though several members of receptor kinases have been shown to play key regulatory roles in plant development (Torii and Clark 2000), it wasn't shown untill recently that one of them, CLAVATA1 (CL1), physically interacted with the small peptide and the presumed ligand CLAVATA3 (CL3) (Trotochaud *et al.* 2000) (Clark *et al.* 1995) (Fletcher *et al.* 1999).

The Aleurone Cells: The aleurone layer is central to cereal seed viability and nutritional value. The outer daughter cells of the first periclinal endosperm division represent initiation of aleurone cell formation surrounding the starchy endosperm (Olsen *et al.* 1998). Most of the grain's oil and protein is located in the aleurone cells, whereas the starch is in the inner cells of most of the remaining endosperm. The number of aleurone cell layers differ between the cereals, with maize and wheat having only one, barley three and rice has a variable number from one (lateral), two (ventral) and up to four to six (dorsal) aleurone cell layers in the grain (Hoshikawa 1993). Aleurone cell identity is possibly initially controlled by clonal events through cell divisions followed by position-dependent events regulated by one or several signals from the surrounding maternal tissue (Olsen 2001) (Becraft and Asuncion-Crabb 2000). In the *Dek1* induced mutants having patchy aleurone the cells that should have aleurone identity given their position get starchy endosperm identity up till at least 22 DAP and can convert back when having the *Dek1* gene restored (Becraft *et al.* 1996) (Becraft and Asuncion-Crabb 2000). This suggests that starchy endosperm identity is the default pathway, and aleurone specificity demand some kind of signal and the ability to respond to this signal. This might be explained by a maternal secreted signal and the outer endosperm cells not having the corresponding appropriate receptor i.e. crinkly4 in this mutant. Both the *Cr4* and the *Dek1* genes are reported necessary to gain aleurone identity. Hopefully cloning and further characteristics of the *dek1* gene might reveal how aleurone cell identity is controlled.

The Strachy Endosperm Cells: Starchy endosperm cell identity might be automatic unless other specificity is received, since knocking out the Dek1 gene cause initial aleurone cells to convert to starchy endosperm cells during endosperm development (Becraft and Asuncion-Crabb 2000).

We have studied the AGPase enzyme, a rate-limiting enzyme in starch synthesis, one located in the strachy endosperm (Opsahl-Ferstad and Rudi 2000). AGPases in higher plants are organized as heterotetramers consisting of two different subunit types, both required for optimal activity (Morell *et al.* 1987) (Okita *et al.* 1990) (Hannah and Nelson 1976) (Hylton and Smith 1992) (Lin *et al.* 1988) (Muller-Rober *et al.* 1992). In barley, two different AGPase isoforms have been purified, one from the endosperm and one from the leaf (Kleczkowski *et al.* 1993a; Kleczkowski *et al.* 1993b). Four different cDNAs encoding the AGPase subunits have been reported (Villand *et al.* 1992) (Eimert *et al.* 1997) (Thorbjornsen *et al.* 1996) encoded by three single copy

genes (Kilian *et al.* 1994). The two different small subunit AGPase transcripts are derived from the single small subunit AGPase gene by alternative promoter usage and differential splicing (Thorbjornsen *et al.* 1996). As a result, the two small subunit transcripts and peptides differ only in their 5`and amino termini, respectively. Northern-blot and RT-PCR analyses have shown expression of the transcripts in endosperm and vegetative tissues, including leaf (Doan *et al.* 1999). Transgenic rice has been used to confirm the activity of the AGPase *BEPS* (Barley Endosperm Pyrophosphorylase Small) promoter in endosperm as well as vegetative tissues. Preliminary results have shown the same expression pattern, with the small AGPase subunit promoter from endosperm, in transgenic rice (unpublished data).

Promoter Analyses in the Cereal Endosperm:We have performed a number of promoter analyses of genes preferentially expressed in the different cell types during endosperm development. Most of our genes isolated from barley are preferentially expressed in the aleurone cells, being the *LTP1*, *LTP2*, *B22E*, while *BEPS* is expressed in the starchy endosperm cells (Thorbjornsen *et al.* 1996; Thorbjørnsen *et al.* 2000). When studying stably transformed cereals, some of the promoters direct the same specific regulation in barley, rice and maize (i.e. LTP2, BEPS) while others show some differences (i.e. LTP1, B22E) between the cereals (manuscripts in prep). We have used stable transgenic rice in order to characterize the barley promoter *LTP2* being preferentially expressed in the aleurone of rice and barley (Fig 1H) (Kalla *et al.* 1994) (Opsahl-Ferstad *et al.* unpublished). The barley *LTP1* promoter shows similar gene expression in barley and rice, yet differences not experienced with the *LTP2* despite their similar expression pattern in barley (Opsahl-Ferstad *et al.* submitted). We further experienced that by using techniques like RT-PCR and stable transgenics, we revealed expression of the barley *LTP1* promoter not earlier detected or reported. Interestingly genes previously thought specifically expressed in one cell type only, are frequently found expressed in additional cells when using more sensitive techniques. Performing a differential screening to detect aleurone specifically expressed genes (Aalen *et al.* 1994), we found most genes to be additionally expressed in the embryo scutellum and it remain to be seen whether any of the genes are specifically expressed. This might be due to the similar roles of these two cell types. So far, none of the regulatory elements reported implicated in aleurone gene expression in maize (Selinger *et al.* 1998) or rice (Wu *et al.* 2000), have led to the identification of similar element in the barley *LTP1* and *LTP2* promoters. Regulatory elements implicated in aleurone transcription in rice include the GCN4, which is lacking in the barley *LTP1* sequence (Skriver *et al.* 1992). We find a part of the barley *LTP2* upstream sequence, not containing previously reported regulatory elements, to give Gus expression in the aleurone in transgenic rice (Opsahl-Ferstad *et al.* unpublished). This indicate that aleurone cell preferred control of transcription in cereals might be more complex, and include mechanisms that are conserved, as well as mechanisms that are species specific. We found two of our aleurone preferentially expressed barley genes (*LTP1*, *B22E*) to have GUS present in the vascular tissues in stems, leaves, roots and seeds in most samples. However, there were exceptions, where GUS was only present in the aleurone and not in the vascular tissues, never the opposite. It is tempting to speculate whether this might be due to unknown transport directed by these pre m-RNAs, as it has been discovered that large mRNA molecules actually are being transported by a possible 'information superhighway' (Strauss 1999) (Xoconostle-Cázares *et al.* 1999). If it is possible that the Gus transcript can be transported, this might explain the quite frequent vascular location of gene sequences regulating reporter gene expression.

Bioinformatics – Additional Tools to be Built and Used in Order to Understand Development and Gene Regulation: The next challenge would be to compare all these promoter sequences in order to possibly identify motifs controlling the preferential regulation. This has been attempted but proven to be difficult, possibly due to many alternative regulatory elements controlling the same developmental stage, cell localization and corresponding regulation or complicated mechanisms not yet elucidated. Comparative genetics and physical mapping between species have shown that even though the gene function of the coding sequences might have been kept through evolution the upstream promoter sequences have changed considerably and the spacing between the genes (Bancroft 2001). Possible identification of common regulatory elements demands highly sophisticated models since we cannot yet define the exact regulatory elements needed for different regulatory specificity (Ohler and Niemann 2001). Not only do we need a certain sequence of the upstream, untranslated region, but also additionally the 5' and 3' UTR, introns and putative enhancer elements further away. Alternative splicing increases the complexity and makes it considerably more difficult to solve the puzzle (Thorbjornsen *et al.* 1996) (Graveley 2001). Combining transgenic experiments with parts of the sequences with the increasing experimental data and newly developed models should make it possible to find some answers. Our experience from the promoters of the *LTP2* (unpublished results) and *ESR* (Bonello *et al.* 2000) genes show that only some 200-300 bases of the upstream sequence are necessary to obtain the regulation expected from in situ hybridisation experiments in accordance with previous reports (Benfey and Chua 1989) (Kalla *et al.* 1994), which is promising for the study and surge of some genes. However, these genes don't have introns, as i.e. the *LTP1* has. When we also experience that the *LTP1* upstream promoter is differentially regulated in barley versus rice, this further indicates that genes with introns might have a more complicated genetic regulation.

Identifying promoter elements containing a specific combination of several regulatory elements demand sophisticated models. So far one has tried identifying common motifs of promoters possibly explaining their regulation through binding the same transcription factors. The available methods are the 'alignment' methods and the 'enumerative' or 'exhaustive' methods (Ohler and Niemann 2001). Using a background models accounting for differences in GC contents makes an important difference. Also a sophisticated model could be constructed from all available and interesting promoters for a given study, but implies specific models constructed in every case very much depending on the information available. The motif-identification problem was one of the prominent topics at the conference on intelligent systems for molecular biology ISMB 2000, and in 2001 this is differentiated to identifying promoter regions, separating real motifs from their artefacts and visualization associations between genome sequences and gene expression data (Blanchette and Sinha 2001) (Chiang *et al.* 2001; Ohler *et al.* 2001). A way of studying the promoters is to group the promoters according to

expression levels and profiles as well as common motifs. Finding the promoters in genomic DNA solarely from the sequence, or by additional information like identifying the TSS (Transcriptional Start Site) and structural properties (bendability and conformation) make significant differences in complexity, probability to find unexpected results and demand critical interpretation of the results. The future will show whether we are able to determine the regulatory regions of eucaryotes and their corresponding transcriptional regulation. The genetic regulation has often changed, while the gene function hasn't. Finally, natural variation of regulatory elements in plant genes could be a treasure box of new paradigms in gene expression. Comparative genomics in plants should have a great future (Messing 2001). When understanding seed development, it would be possible to modify the amount of some of the cell types and thereby change seed content. Combining increased amount of i.e. aleurone cells with controlled spaceal expression of wanted gene products open numerous opportunities in molecular, industrial and conventional farming (Daniell *et al.* 2001).

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