

# UPSTREAM FUNDAMENTAL RESEARCH IN BAMBOO - POSSIBILITIES AND DIRECTIONS<sup>1</sup>

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## ABSTRACT

The development of bamboo as a multipurpose plant on world-wide scale will critically depend on basic research. About basic biology and genetics of bamboo very little is known and basic research is almost non-existent. With our research we concentrate on many different fields and we want to contribute to a better understanding of bamboo. While much of this basic research is not directly valorisable within our plant production, we consider this approach as valuable and strategic in the long term, to improve the economic added value of bamboo, as a plant. Our cutting edge research has led to a number of important breakthroughs and has been the key to our success in mass scale propagation. Main research topics in tissue culture, physiology and genetics are discussed.

- Identification and isolation of endogenous molecules in bamboo has allowed to improve quality and rooting of tissue culture plants.
- Nodule culture and somatic embryogenesis in mature bamboos have been pioneered.
- Flowering has been induced in mature bamboos (tropical and temperate) *in vitro*.
- Use of molecular markers (RAPD, transposon based primers, and AFLP) are used mainly for precise identification, to assess natural variability and for early assessment
- Transposons are possibly involved in somatic mutations in bamboo and in flowering. Ac-like transposable elements are indeed present in the bamboo genome.
- Estimation of DNA content has revealed basically two groups of woody bamboos, namely tropical and temperate bamboos, although DNA content per se is not related to size. The DNA content per chromosome was found to be comparable to rice.
- In *Fargesia murieliae*, flowering monocarpically, reversion of flowering was induced.
- The role of drought and high light intensity on the development of flowering is linked to oxidative stress; important defence systems of the plant are immobilised.
- In leaves of flowering *Fargesia murieliae* DNA is replicating. The possible role of this strange phenomenon is that DNA may serve as source for production of cytokinins.
- The description of inflorescences of bamboo has been problematic in the grass family. We have proposed a more comprehensive approach, using the typology of Troll.

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## INTRODUCTION

Bamboo is a very important plant, providing livelihood for millions of people around the globe. But its actual importance and the research efforts spent on bamboo is highly imbalanced compared with other agricultural crops. In my view basic research in bamboo is very, very important for the future of bamboo. We, as bambusiasts, cannot in general answer simple questions that consumers ask ; we know approximate figures about yields, we can tell that bamboo is a fantastic plant, and we often exaggerate its potential because of our enthusiasm, we know that certain clones can resist more to drought than other. Questions like this can be answered more easily for plants like poplar, pine, Eucalypts, corn, canola (rapeseed), wheat and so on. The main reason of course is that more research efforts and money are spent in these plants, and very little in bamboo.

We must realize that there is absolutely no tradition of fundamental research in bamboo. While bamboo is a very important plant which has played a very important role in the history of mankind, basic research, at least to the level where research is being conducted in other agronomic grasses like wheat and corn, or other crops and plants, has been almost nonexistent. Corn e.g. has been used in fundamental research on morphology (e.g. roots), genetics (transposons, classical genetics) and even now, in the *Arabidopsis* era, corn continues to play a crucial role. Wheat on the other hand has been serving as an example and tool to study polyploid genomes. Fundamental research at all levels has been extensive in both plants. In bamboo, applications and applied research have always been given priority. The added value of bamboo is as a product, downstream. And if research is performed, it is mostly in isolation; concerted research is uncommon.

Bamboo is a difficult plant in many respects. It does grow like a weed, but it is not easily « controlled », as we, agriculturists, would like. For instance, it is impossible at present to set up breeding programmes, since we do not understand flowering, nor are we able to control it. Large scale propagation is difficult, because of the lack of excellent propagation systems, although on the production side of bamboo research and research efforts are gaining some momentum. Both examples are upstream of the bamboo production-transformation chain.

What I will tell you about, is upstream research, the « real » basic biological research of the plant proper. In this field we have built up considerable experience, and I want to give a short overview of our findings so far, which directions we, and hopefully others, can proceed, learning from our experiences. Basically all the research we do is oriented at genetic improvement of bamboo. The research may be academic or very basic as long as it fits in some way with our strategy, which is aimed at genetic improvement of bamboo and mass scale propagation of elite genotypes.

We have been focussing on both physiology and genetics of bamboo, and some of our findings have already been translated directly into practical and commercial (Gielis and Oprins, 1998 and this congress), others are just preliminary experiments, that will hopefully be useful in the long run. While some of this research may look more or less academic, it has been a way to gain « understanding » of bamboo, or better, to develop a feeling for bamboo. Many of the research strategies adopted are quite original, and do not depend on findings in other plants. By using a mix of strategies, I believe, may we catch up some of the lost time, and transform bamboo as a plant into a plant with high economic added value.

# PART 1 : GENETICS OF BAMBOO

## PROBLEMS AND APPROACHES

When we want to study the genetics of bamboo we face enormous difficulties simply because we cannot make hybridisations as easily as we would like. Natural hybrids do occur, and artificial hybrids have been made, but such controlled crosses are very rare, and do not allow to make extensive studies of genetics. These difficulties are even worse since in no case we have F<sub>2</sub> populations, let alone that back crosses can be made. Seed orchards (Banik, 1995), *in vitro* flowering or other means to induce or control flowering are very important in the future, if we want to achieve "full domestication".

Many of the "characteristics" of bamboo such as special anatomy and branch complements, turn out to be lost in wide crosses with sugarcane (Rao et al., 1967). It seems that the "special bamboo characters" are the result of polyploidisation. And this makes the study of genetics even much more complicated. Moreover, natural populations are very heterogeneous and heterozygous. Modern biotechnological techniques however, such as molecular markers, do allow to attack some problems in a well defined way, although we must remain modest in our expectations. It will take quite some years before the agricultural value of bamboo is balanced by fundamental research.

## MOLECULAR MARKERS

Given the long feedback times to monitor and select elite genotypes in bamboos, and given that identification of genotypes is very difficult, the use of molecular markers, may turn out to be very useful for different purposes. As a tool, molecular markers will save lots of time, although careful consideration must be made when and where to apply which method. In any case using molecular markers without reference to field studies is not very useful.

In the last ten years we have already witnessed several generations of molecular markers, which become increasingly precise, but also require a new approach every time. Starting with isozymes and proteins, in the second half of the eighties restriction digestion and visualisation of DNA fragments obtained by restriction digestion (RFLP) were used widely, also in bamboo. The big boom of molecular markers came with PCR-technology, with Random Amplified Polymorphic DNA and related techniques (for review of molecular markers in bamboo, see Gielis et al., 1997). RAPD have been used in *Yushania* (Hsiao and Riesebergh, 1994) and *Phyllostachys* (Gielis et al., 1997d; Ding, 1998). Based on a combination of RAPD markers and morphological markers (Ding, 1998), it was concluded that *P. bissetii* and *P. nigra*, among others, do not belong to the section Heteroclada, as had been suggested (Gielis et al., 1997d). Other problems which could be resolved by Ding using RAPD, was the conspecificity of *P. violascens* and *P. praecox* on the one hand, and of *P. humilis* and *P. varioauriculata* Li and Wu on the other.

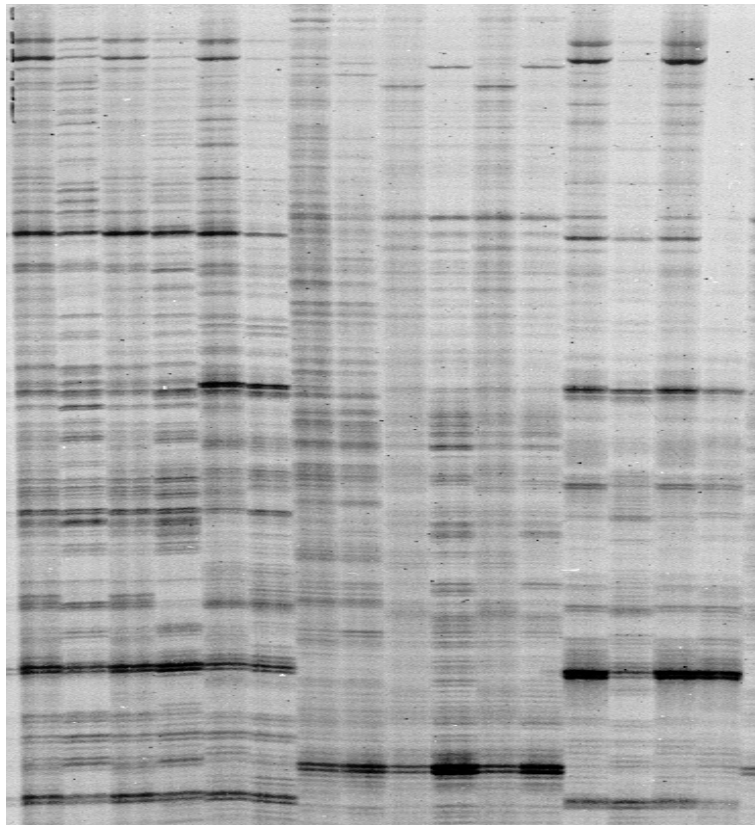
But nowadays, RAPD's have become out of date. Amplified Fragment Length Polymorphisms AFLP™, a combination of restriction digestion and PCR amplification (Vos et al., 1996) and SAMPL, a combination of AFLP™ and Single Sequence Repeats SSR, are the new generations of arbitrary molecular markers (Figure 1).

	<b>RAPD</b>	<b>CAPS</b>	<b>SSR</b>	<b>AFLP</b>	<b>IRA</b>	<b>SAMPL</b>
Principle	Random PCR amplification of genomic regions	PCR amplification, restriction digestion	PCR amplification of microsatellites	Restriction digestion, adapter annealing and selective PCR	PCR of inter-SSR regions	Restriction digestion, adapter annealing, PCR with AFLP primer and compound SSR
Nature of polymorphisms	Base changes, insertions, deletions	Base changes, insertions, deletions	Variation in repeat length	Base changes, insertions, deletions	Insertions, deletions	Base changes, insertions, deletions, repeat length
Level of Polymorphisms	Medium	Medium	Very high	Medium	Medium	High
Abundance	Very high	High	Medium	Very high	High	High
Dominance	Dominant	Codominant	Codominant	Mixed	Mixed	Mixed
Multiplex ratio	5-20	1	1	50-100	20-100	10-50
Initial DNA amount required	20 ng	50ng	50-100 ng	0.5-1 µg	50-100 ng	0.5-1 µg
Sequence information required	No	Yes	Yes	No	No	No
Costs	Low	High	High	Medium	Medium	Medium

Figure 1 : Comparison of PCR-based molecular markers (Breyne et al ., 1997)

In one single lane of an AFLP™ gel, up to 100 different highly reproducible AFLP™ fragments can be visualised (compared to about 7-10 fragments in RAPD) corresponding to about 1/32000 of the total genome, representing about 0.0003 % of the total genome. One restriction enzyme combination and 6 selective nucleotides allow about 4000 different experiments, theoretically yielding a marker every 50 kb (assuming 10% polymorphism). Given the reproducibility and the information that can be obtained, the working costs of AFLP™ are quite low, even compared to RAPD. The current research topics in which we use AFLP™ are:

- Identification of *Phyllostachys*, *Bambusa* and *Fargesia* species and cultivars and generation of databases (Figure 2).
- Customer service: DNA fingerprinting for identification and cultivar protection can be done at request
- Study of natural variability and genetic diversity in *Gigantochloa scortechinii*. This study is conducted in co-operation with field research and anatomical research and multivariate analysis should yield very useful information, regarding the link between morphological, anatomical and molecular data (EC-project ; co-operation with FRIM, Malaysia).
- Based on 450 AFLP fragments, *Bambusa vulgaris* and *B. striata* differ by 5 bands (about 1.2% of difference). It is clear that *B. striata* is not a somatic mutant of *B. vulgaris* and is rather a separate species as was proposed Bennet and Gaur (1990)
- Early assessment and the construction of genetic maps in *Dendrocalamus strictus* and *Dendrocalamus giganteus*.
- Comigrating Rice AFLP fragments should allow to use information from rice to study bamboo.



1al 1bl 1as 1bs 2a 2b 3at 3bt 4ao 4bo 4ay 4by 5ay 5by 5ao 5bo

Figure 2 : AFLP pattern in bamboos ; the influence of DNA isolation procedures (Gielis et al., 1997e)

- Genotypes
  1. *Phyllostachys edulis*
  2. *P. aurea* 'Koi'
  3. *Bambusa ventricosa*
  4. *Bambusa vulgaris*
  5. *Sasa veitchii*
- DNA isolation procedures
  - a : Cesiumchloride
  - b : CTAB
- Age of leaf material
  - o = old and y = young
  - l = large and s = small
  - t = from tissue culture

AFLP is currently widely used also in human genetics. It is highly reproducible, but quite surprisingly, the AFLP™ fragments seem to depend on the DNA isolation method (Figure 2; Gielis et al., 1997e), which does affect the sampling of material in the forest. Care must be taken in sampling leaf material, as well as in isolation and purification of DNA. A future direction to study natural variability in bamboo is SAMPL in which AFLP and micro-satellites are combined in one technique. Micro-satellites have already been studied in bamboo (Zhao and Kochert, 1993). In SAMPL the level of polymorphism is very high, higher even than in AFLP.

## TRANSPOSONS IN BAMBOO

Research also focusses on mutations in bamboos. Somatic mutations in bamboo are highly valued in horticulture, and are an intriguing research subject. There is a huge variation in stem colour and stem morphology and there is some reason to believe that these somatic mutations are caused by transposition events (« mutable genes », Okamura, 1986). Like in corn and many other plants, transposition can lead to reorganisation of the genome, under conditions of stress (McClintock, 1986). Most importantly, it has been observed in some bamboos, that an increased rate of mutations precedes flowering. In some unknown way, genomic rearrangements may occur or even be the cause of flowering. So there are very good reasons to study transposons in bamboo. The somatic mutants as observed in stems are important phenotypic markers, but biotechnological tools allow to study the transposons and transposition at the molecular level.

Most probably there are a large number of different transposons present in the bamboo genome. We have chosen two approaches to study transposons in bamboo. A first approach

was to use primers based on sequence information from the 4.5 kb Ac9 transposon from maize. Several primer combinations were used and we could show that several copies with considerable homology to the original Ac9 transposons were present (Figure 3).

Eventually it turned out to be a useful and relatively cheap method to identify bamboos, at the species level (Gielis and Sormann, 1997). In Figure 3 one can see clearly the difference between *Fargesia murieliae* and cultivar 'Harewood' on the one hand, and *Fargesia nitida*, on the other, under different annealing temperatures.

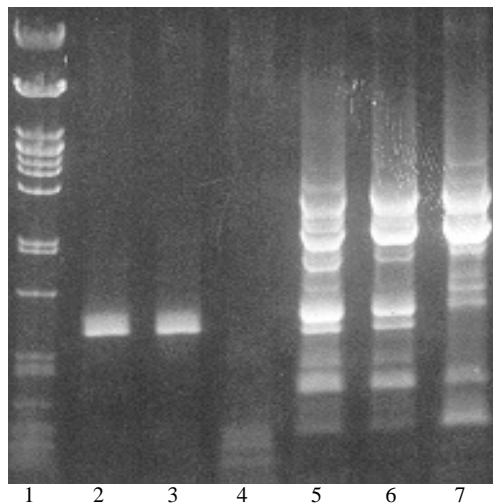
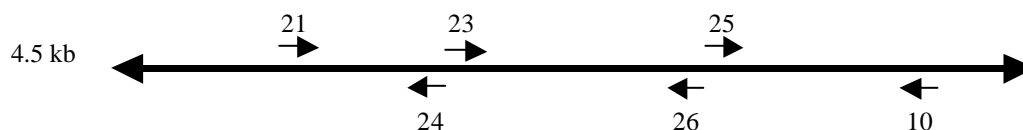


Figure 3: Profiles obtained with PC3. Lane 1: MW marker; Lanes 2 and 5: *Fargesia murieliae* 'Harewood'; Lanes 3 and 6: *F. murieliae*; Lanes 4 and 7: *F. nitida*. Annealing temperatures are 50/55°C in lanes 2-4, and 45/50°C in lanes 5-7.

The primer combinations used all amplify a specific region of Ac9. Primer combinations : PC1 : 21-10 :3700 bp / PC2 : 23-26 : 1250 bp / PC3 :21-24 :850 bp/ PC425-10 :1200 bp. Annealing temperatures : 45°C 10 cycles + 50°C 25 cycles, or : 50°C 10 cycles + 55°C 25 cycles

21. CAAGC TGATT GCTGA ACACC	Position	911
23. AGTAC GATGA AGTGG TTAGC		1759
24. CTTTA GGCTA ACCAC TTCATCG		1763
25. TCTAG TTGTA GTCCT TCAGC		3032
26. GGAGC TGAAG GACTA CAACT		3035
10. CTTGG GCTCT TGGCT AACAT		4211



In another approach an AFLP primer was used to isolate transposons (Peter De Keuckeleire and Tom Gerats, VIB, Laboratory of Genetics, Gent, in preparation). AFLP markers are basically arbitrary markers for which no preliminary sequence information is required. But it is also possible to use AFLP for detection of very specific genomic fragments with known sequence, for example transposons. In this respect primers were designed specifically to detect *Activator*-like transposons in *Petunia*. Using this primer with other plants, fragments could also be detected in bean, aza lea, brassica and bamboo.

The same fragment was detected in the three bamboos tested, namely *Bambusa vulgaris*, *Sasa veitchii* and *Phyllostachys edulis*. The fragment of *Bambusa vulgaris* (*hATbv1*)

was sequenced and shown to be homologous to members of the *hAT* superfamily of transposons, to which also *Ac* (corn), *Tam3* (*Antirrhinum majus*) and *hobo* (*Drosophila*) belong. The homology was as high as 60% in some regions, which conforms also to earlier findings of *Ac*-like sequences in *Bambusa multiplex* (Huttley et al., 1995).

It is very likely that many other transposons are present in the bamboo genome as well. It is however, something entirely different to prove that transposition occurs and is indeed responsible for the observed effects. One approach is to study via reverse transcription PCR (RT-PCR) if this *hAT*-like transposon is transcriptionally active in bamboo in different somatic mutants. Also, the question how transposons would act is important, since they are known to act on the genome in quite a number of different modes (Wessler, 1998). This is a long term research project, but we have shown that the tools are there to study it.

### ESTIMATION OF DNA CONTENT

Bamboos are polyploids; temperate bamboos have 48 chromosomes, tropical ones have 72 chromosomes, so they are tetra- and hexaploids respectively, assuming a basic chromosome number of  $x = 12$  in grasses. The chromosomes of tropical bamboos are very small and the karyotypes of temperate bamboos are very complicated (Kondo, 1964). Also given the lack of breeding systems, it seems almost impossible to do any serious study on the genome of bamboo.

But in plants, and more specifically in grasses, large parts of the genome are collinear, that is, genes are arranged in similar way on chromosomes, and there is quite a large degree of genome conservation. If this were true, one might exploit the knowledge generated in e.g. rice, to study the genetics of bamboo, since rice and bamboo are very closely related within the grass family. But as a first step it is necessary to estimate the complexity of the genome. To this aim flow cytometry was used, which yielded some very interesting results (Gielis et al., 1997c).

- There are two main groups in bamboo, the temperate bamboos with DNA contents of 4-5.5 pg/2C, and the tropical bamboos with about 2.5-3.2 pg/2C.
- If calculated per 12 chromosomes (one «complement») the DNA content of tropical bamboos is very similar to that of the haploid genome of rice ( $x = n = 12$ )!!!
- Genome size has little connection to size of plants, as *Phyllostachys* has the lowest DNA content in temperate bamboos, and the herbaceous bamboo *Lithachne humilis* (3.69 pg/2C) has a value intermediate between tropical and temperate bamboos
- Endoreplication of DNA in leaves of flowering *Fargesia murielae* was observed.
- The results also suggest that polyploidy has been one of the major driving forces in the evolution of woody bamboos.

So we learned that rice and bamboo are very close regarding genome complexity (also chromosome size, for tropical bamboos). In our AFLP experiments also rice was used, and some bands were comigrating with bamboo fragments (Gielis et al., unpublished results). Since AFLP is a very precise method these fragments have a high chance of being the same DNA sequences. So in some ways it will be possible to use results from rice studies in bamboo, with this restriction that bamboos are polyploids. Wide crosses between rice and bamboo (Zhang and Ma, 1991) should provide ideal material for such studies.

## PART 2 : FLOWERING OF BAMBOO

“One of the great mysteries in botany”, as flowering of bamboo is usually advocated to the world. In other respects, flowering is considered a catastrophic event, destroying large areas and whole populations, depriving panda’s from food, and people from income. However my personal view has always been that a rational view of flowering in bamboos would also imply a demystification, since many of the ‘tales’ are very often based on non-proved facts and assumptions. It has always been my belief that in some way we will be able to control flowering in bamboo. In fact if we want to turn bamboo into a real agricultural important plant we must be able to control flowering, either to put up breeding programs or to avoid catastrophic losses in agroforestry.

Our research in bamboo flowering has focussed on two aspects. One aspect has been the “monocarpic” flowering of *Fargesia murieliae*, in which morphological and physiological aspects have been studied, and the other has been flowering in tissue culture. Flowering was observed repeatedly in mature bamboos in tissue culture which opens up enormous possibilities to achieve our aims of controlled hybridisations.

### EXPERIMENTAL REVERSION OF FLOWERING IN *F. MURIELIAE*

*Fargesia murieliae* is one of the most important garden bamboos, which started flowering heavily in the early nineties, all over Europe. Almost all plants, if not all, have burst into flowering, resulting in most cases in the death of the plant. As all these plants were propagated via vegetative propagation, starting with one plant only in the second decade of the 20th century, this case is probably the best proof ever for any induction theory based on ontogenetic age.

Flowering plants of *Fargesia murieliae* were placed under different treatments in 1994, to study the effects of drought, light intensity and temperature. In one of the treatments, with higher temperatures and low light intensity, reversion of flowering was observed. Inflorescences that were already formed, developed into vegetative shoots. Indeed lemmata developed into normal leaves with expanded blades and the lateral shoots did not develop into palea and flower (Figure 4).



Figure 4: development of vegetative shoots in the short paracladial zones

Now, after four years, the plant is growing vegetatively again with many delicate shoots, arising from the basal parts of the plant (Gielis and Goetghebeur, 1995; Gielis et al., 1999). These experiments showed that even monocarpic flowering of species with semelauctant inflorescences can be reversed as is known in bamboos with leptomorph rhizomes (Ueda, 1960). The inflorescences proper can reverse from flowering to vegetative states, and buds proximal to the inflorescences can develop into innovation shoots.

## **OXIDATIVE STRESS PROVIDES THE LINK BETWEEN FLOWERING AND ENVIRONMENTAL CONDITIONS**

In our treatments it was observed that the development of flowering can be modulated to a great extent by environmental factors. More in particular, we found that adverse conditions such as drought and high light intensity generate excessive oxidative stress in spikelets, which results in an increased activity of ascorbate peroxidase POX and superoxide dismutase SOD, as well as mobilisation of different isoforms of SOD.

But the same stress conditions also immobilise one of the most important defence mechanisms, namely catalase, the enzyme which converts hydrogenperoxide into water and oxygen in glyoxisomes ( $H_2O_2$  as intermediate product of fatty acid degradation) and peroxisomes ( $H_2O_2$  as intermediate product of photorespiration). This leads to excess of hydrogenperoxide, which can cause damage to biological molecules. So this gave a definite proof that oxidative stress provides the link between environmental conditions and biochemical events associated with development of flowering and senescence of the inflorescences.

Recently experiments under high ozone conditions induced flowering in bamboos (Tesche, pers. comm.), confirming our hypothesis that oxidative stress is involved in induction and development of flowering. This also allows us to formulate management considerations for silviculture.

## **DNA AS A SOURCE OF CYTOKININS?**

One of the most intriguing aspects of the development of flowering in *Fargesia murieliae* was the finding that in flowering plants substantial endoreplication of DNA was observed, more particularly in green spathes, subtending the racemose inflorescences (Gielis et al., 1997b). This means that during the development of flowering, nuclei in green leaves start to double and triple or quadruple their DNA content, for some unknown reason. Such endoreplication was also observed in older leaves of *Lolium* at the end of the growing season (De Loose et al., 1994). In our experiments endoreplication is not observed in vegetative leaves, nor in leaves on plants in which flowering is reverting (the lemmata described above). In those leaves only 2C peaks were found. But in flowering plants, what also was found is that in the spathes, many nuclei have less DNA than the expected 2C level. So in fact, the nuclei loose DNA, and this is observed in green leaves, without any visible sign of senescence, and prior to the generation of oxidative stress. So this endoreplication of DNA is a very early marker of senescence.

The biological function of this is totally unclear. The hypothesis I follow at present is that this DNA may serve as a source of cytokinins. Indeed, if DNA is degraded it sets free a

large pool of AMP which may directly be converted into cytokinins by AMP- $\Delta^2$ -isopentenyl transferase. These cytokinins, in a nice positive feedback loop trigger DNA synthesis. The function of cytokinins is clear, namely retardation of senescence (although cytokinins are also involved in the induction of flowering. A very interesting hypothesis, since it provides a possible pathway for synthesis of cytokinins in leaves directly (Gielis et al., 1999).

## **IN VITRO FLOWERING IN BAMBOOS**

The first reports on tissue culturing flowering of bamboos (Nadgauda et al., 1990; Rao et al., 1990) caused great excitement. It opened up the possibility of controlled flowering that can be used for breeding of bamboo. Since then *in vitro* flowering has been observed in many types of bamboos.

In some cases the induction can be well controlled experimentally, in other cases only some observations have been reported. But once inflorescences are identified in tissue cultures, subsequent isolation and subculture of these inflorescences can be used to establish monocultures of inflorescences.

### *In vitro* flowering in seedlings

- In embryogenic calli of *Dendrocalamus strictus* and *Bambusa bambos* (Rao et al., 1990)
- In shoot cultures of *Bambusa bambos* and *Dendrocalamus brandisii* (Nadgauda et al., 1990)
- In shoot cultures of *Dendrocalamus hamiltonii* (Chambers et al., 1991)
- Flowering in *Bambusa vulgaris*, *Dendrocalamus strictus* and *D. giganteus* (Rout and Das, 1994)
- In shoot cultures of *Dendrocalamus asper* (Arya and Arya, 1996)
- In somatic embryos of *Bambusa oldhamii* (Chang et al., 1997)

### *In vitro* flowering in adult bamboos

- Shoot cultures of *Bambusa nana*, *B. bambos*, *B. multiplex*, *B. brandisii*, *Dendrocalamus membranaceus* and *Cephalostachyum pergracile* (Prutpongse and Gavinlertvatana, 1992)
- Shoot cultures of *Bambusa ventricosa* (Gielis, 1995)
- In shoot cultures of *Dendrocalamus asper* (Arya and Arya, 1996)
- In shoot cultures of *Bambusa ventricosa*, *Bambusa multiplex*, *Phyllostachys* sp. and *Chusquea* sp. (Gielis et al., 1997b)
- *Dendrocalamus longispathus* (Sanjay Saxena, pers. comm.)
- In shoot cultures of *Bambusa oldhamii* (Chang et al., 1997)
- In shoot cultures of *D. brandisii*, *D. latiflorus* and N°7 hybrid (Zhang and Wang, 1998)

The induction of flowering in mature bamboos gave the definite proof that the induction of flowering has a physiological basis. *In vitro* flowering of mature bamboos was also important for another reason, namely that flowering can be induced in elite genotypes. In

seedlings, one does not know the genotype and phenotype (unless plants are planted in the field and monitored).

Possibilities opened by *in vitro* flowering of bamboo are (Rao and Zamora, 1995; Gielis and Debergh, 1998):

- controlled induction of flowering in economically important bamboos
- induction of flowering in hitherto non-flowering bamboos (taxonomy)
- hybridisation (interspecific / intergeneric or wide crosses) and seed production *in vitro*
- production of pollen grains and/or ovaries, which can be isolated and stored
- hybridisation techniques *in* or *ex vitro*, with reproductive cells or organs from one parent obtained *in vitro*
- embryo cultures for crosses with low success rates
- generation of haploids and doubled haploids for breeding purposes
- use of haploids for cytological and molecular studies
- use of any part of the pseudo-spikelets for plant propagation, via nodule cultures, organogenesis or somatic embryogenesis.

Almost 10 years after the first report on *in vitro* flowering in bamboo, most possibilities presented above have been experimented. Although some positive results have been obtained, practical and commercially exploitable results have not been reported yet. Of the above identified possibilities, the most promising ones are those involving hybridisation under controlled conditions. However detailed studies and results are very scarce. Most often the development of pseudospikelets yields normal florets with male and female reproductive organs. These organs are smaller than those observed under *in vivo* conditions (Rao and Zamora, 1995; Nadgauda et al., 1997b). The main problems for *in vitro* hybridisation seem to be pollen viability and synchronisation of flowering. Pollen viability is always much lower *in vitro*, possibly due to defects in wall formation (Nadgauda et al., 1997b). Seed setting has been observed (Nadgauda et al., 1990; Rout and Das, 1994) but at low percentages, and apparently only when many flowers were open at a particular moment (Nadgauda et al., 1997b). It is necessary to intensify research in this field. Many hurdles still need to be taken before the methods really become applicable at agricultural scales.

From a scientific point, the search for causative agents is highly interesting. It is evident from *in vitro* flowering observations that the induction of flowering in bamboos has a strong physiological basis. But while it is possible to induce flowering, the search for the causative agents remains very difficult. Cytokinins and sugars are involved in some way (Nadgauda et al., 1997a; Chambers et al., 1991), but these are involved in the induction of flowering in many plants (Scorza, 1982; Bernier, 1996). Nadgauda and co-workers obtained flowering in up to 70% of the seeds used, when the seedlings were cultured in bioreactors. But plants cultured in liquid also show an enhanced level of oxidative stress (Gielis, unpublished results).

While the efficiency of flowering induction in bamboo tissue cultures can gradually be favoured by improved knowledge and modification of some physical and/or chemical *in vitro* parameters, in our opinion the search for a single factor or few factors as a general causal agents of flowering, is not a good strategy. Earlier attempts in this direction have not yielded any result. For example: flowering has been observed in many other plants in tissue culture systems, already very early in the history of tissue culture (Scorza, 1982 for a review). Flowering was reported in entire plants from seeds, from preformed meristems, on stem internodes, leaf discs, hypocotyl, petiole and tendril segments and epidermal and subepidermal sections. For induction and/or evocation of flowering sugars are always

necessary and cytokinins are also a very common requirement. But neither sugar, nor cytokinins, alone or in combination, provide the ultimate cause for flowering.

*In vitro* flowering is thus a widespread phenomenon, but the underlying causes have not yet been identified. It is interesting to compare this *in vitro* flowering of bamboos with the floral transition phase in the plants *Lolium temulentum*, *Sinapis alba*, *Arabidopsis thaliana* (LD plants) and *Xanthium strumarium* (SD plant). After induction of flowering *in vivo* as a result of photoperiodic induction, the level of sucrose in the phloem sap and at the shoot apical meristem increases, at least temporarily (Bernier, 1996). Its possible function seems to be mainly stimulation of metabolic activity. Also at the floral transition phase, cytokinins are transported to mature leaves and further to the shoot apical meristem where they exert several effects, mimicking changes associated with the transition to flowering they cause an increase of the mitotic and DNA synthesis indices, by shortening the duration of the S and G<sub>2</sub> phases (Bernier, 1996 and references therein) and the breakdown of vacuoles into smaller units. But yet, combined treatment of sugars and cytokinins are unable to cause the floral shift in *Sinapis alba*. Application of Benzyladenine to bamboos *in vivo* also did not result in the induction of flowering (Nadgauda et al., 1997a)

So in other studies on *in vitro* and *in vivo* flowering too, components as sugars and cytokinins have been identified as important components, but still a number of other factors are involved, both promoting and inhibiting, which remain unidentified. Given the complexity of the flowering process, it seems unreasonable at present to expect that the secrets of flowering will be unravelled only based on tissue culture experiments. Indeed, the process is much more complicated than a simple transition from the vegetative to the flowering state.

As an alternative to physiological studies, molecular studies may be used and findings in other plants may possibly help to understand the molecular mechanisms underlying flowering. In bamboos flowering is observed in shoot cultures as well as in somatic embryos developing from callus tissue of *Bambusa bambos* and *Dendrocalamus strictus* (Rao and Rao, 1990), and in callus tissue of *Bambusa oldhamii* (Wei-chin Chang, 1997). This bears some resemblance to the *EMF/emf* mutants in *Arabidopsis thaliana* (Sung et al., 1992).

In *emf* mutants too, flowers develop from callus tissue, and no vegetative shoots are formed. The hypothesis was formulated that the dominant *EMF*-gene was necessary for the suppression of flowering, thereby suggesting that the reproductive state is actually the ground state in flowering plants. Its action would be most pronounced during embryo and seedling development, gradually diminishing until the ontogenetic age of flowering has been reached

Similar genes may occur and be active in bamboo as well, but due to the polyploid genome, in more than one copy. Following this hypothesis the *EMF*-like gene is expressed in the vegetative state. The action of this dominant gene could be suppressed due to abnormal conditions which favour flowering, such as tissue culture conditions, hence flowering of shoots and somatic embryos. This hypothesis is even more attractive by the finding in *Bambusa tulda* that the character of precocious flowering in seedlings at about 18 months is inherited in at least 5 successive generations (Banik, 1987). Precocious flowering genotypes in a seedling population would be recessive mutants for an *emf*-like gene. Molecular approaches may be complementary to physiological approaches and may or will improve our understanding of flowering. Many findings in other plants are very significant for bamboo.

## THE PLASTICITY OF FLOWERING

One main line of thought in our work on flowering is our belief that flowering is flexible, and convertible. This is observed in tissue culture, and as in *Fargesia murielae*, flowering can be controlled and reversed in monocarpic bamboos as well. While in nature monocarpic flowering may be the rule in many bamboos (Janzen, 1976; Banik, 1995), cultural conditions can control flowering to a large extent, precisely what is needed for full domestication of bamboo. *In vitro* flowering and vegetative states are convertible quite easily, so that the line between flowering and vegetative may be very thin.

The plasticity of flowering we find throughout also calls for a novel approach to describe inflorescences. The relation between monocarpic flowering and semelauctant inflorescences on the one hand, and iteroparous flowering and iterlauctant inflorescences on the other, can no longer be held, at least not as a simple approximation. Indeed reversion of flowering is possible also in bamboos with semelauctant inflorescences and innovation zones can be observed either in the inflorescence proper or by the development of proximal buds into vegetative shoots rather than into inflorescences.

The novel approach to cope with this plasticity can be found in the typology of Troll, used in many other plants as well, including grasses. In bamboos this typology was first used on *Maclurolyra* (Calderon and Soderstrom, 1973) but subsequent treatments have been very confusing and the ultimate unit of the typology, the florescence, has been defined in many different ways. This situation has been clarified (Gielis and Goetghebeur, 1997). Here we defined the florescence as starting distally on the axis, the terminal part of the spikelet axis and the fertile lemma's, and, if present, the sterile lemma's and empty glumes.

These sterile lemma's or empty glumes are interpreted as inhibition or repression zones, but are included in the florescence as in other grasses (Vegetti and Weberling, 1996). In pseudospikelets, the proximal zone with gemmiparous bracts, as well as the more proximal empty glumes and the prophyll, have to be described as inhibition or supplementing zone, depending on the actual development. The actual development is crucial here: we no longer need a descriptive typology of inflorescences that is suitable for taxonomists, but we need a flexible approach to deal with reversion and modulation of flowering.

## CONCLUSION

Our fundamental research has yielded some very important results. Most important in the short term of course has been the valorisation of research on the physiology of bamboo into our tissue culture production, with a capacity of up to 3 million bamboos per year (Gielis and Oprins, 1998, and this congress). But also other research results are very interesting and promising. Most of the research is ongoing, but I believe that the results we obtained so far are very useful in middle and long term. It is not at all straightforward for private companies to conduct so much fundamental research, but in a way we had to. And now some important results of our research have been implemented in production.

Bamboo is one of the most valuable plants nature has given to mankind. But to use its full potential, more fundamental research is needed urgently, to lay the foundations for the future. And so are concerted actions. Concerted research avoids redundant research, and allows to define specific goals, from which everyone can benefit.

Bamboo has never been a model plant for any field of research. We are always lagging behind for at least ten years or so. Only in recent years this has slowly been changing. In some ways bamboo now has become crucial or pivotal in some fields of research. It is not so that bamboo will substitute other model plants like corn or *Arabidopsis*, rather in many ways some of the discoveries that were made in bamboo, turn out to be extremely useful for studies in other plants as well, as they provide new insights. Some examples:

- Endoreplication of DNA during the development of flowering and its relation to senescence in monocarpic flowering (Gielis et al., 1997c).
- The discovery of exponent moderation in plants, possibly one of the major biological principles, finds its basis in hollow internodes and square bamboos (Gielis, Exponent moderation in plants, this congress)

Alternatively, studies in bamboo have shown that in certain ways bamboo provides the missing link in some fields of biological studies, enlarging the understanding of the versatility of concepts:

- The crucial position of bamboos in phylogenetics of the grass family (Clark, 1997).
- The relation of morphological versatility in flowering of bamboo and its relation to inflorescence typology in grasses (Gielis and Goetghebeur, 1995; 1997; Stapleton, 1997).

These few examples are but first glimpses of hope, but it may get bamboo in focus of other researchers.

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