Retrotransposon-based Variability in Pinot Noir Clones revealed by S-SAP

Masterthesis

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I. Introduction

Grapevine (*Vitis vinifera* L.), economically one of the most important crop plants, comprises multiple cultivars and clones. The identification and characterization of grape varieties has always been an intrinsic concern for agriculture as well as for breeding research programs. Traditionally, ampelographic and ampelometric methods, both based on morphological characteristics, have been implemented for distinguishing grapevine cultivars. These time-consuming and cost intensive analyses have revealed to be unprecise and unreliable in many cases. Limitations have occurred concerning the objectivity and the reduced period of applicability. Many morphological characteristics such as fruit features are not visible until late developmental stages since perennial grapevine shows a long juvenile period. Moreover, distinctive traits among closely related accessions are often marginal whereby classical ampelography has resulted in insufficient or even unsuccessful differentiation (Lavi et al. 1994, Thomas et al. 1998).

Promising aids for the characterization and classification of grape cultivars and clones, complementing the classical procedures, are molecular marker systems based on differences on the DNA level. So far, several markers have provided encouraging results in clarifying relationships among registered grapevine cultivars (e.g. Regner et al. 2000). As in other vegetatively propagated plants, DNA marker analyses have demonstrated and confirmed the presence of clones. Grapevine clones, which demonstrate only minor genotypical and phenotypical differences (e.g. berry cluster), are generally difficult to distinguish by classical ampelography as well as by molecular techniques.

An encouraging marker system for the distinction of closely related clones, however, has been the S-SAP method. Based on the occurrence of retrotransposons within the plant genome, this method exceeds most other PCR approaches by revealing higher levels of polymorphisms. Retrotransposons are characterised by their capability to translocate and change their genomic location, whereby they generate transpositional polymorphism. So far, three grapevine retrotransposons have been classified but more elements are expected to exist.

Their ubiquitous distribution within most plant genomes reflects a significant role in the evolutionary history of plants, and thus also in the evolution of grapevine.

A promising model for investigating clonality in grapevine is the ancient Pinot cépage (Hocquigny et al. 2004). Previous studies have revealed their clonal relatedness and their high genetic similarity among each other, also indicating their origin in asexual reproduction (Ye et al. 1998, Regner et al. 2000). Compared to other grapevine cultivars they are characterised by a high phenotypic diversity, originated by spontaneously occurring mutations. Several pale coloured mutants have emerged from the red grape Pinot noir. Pinot gris has been identified as periclinal chimera resulting from somatic mutation at the berry colour locus (Walker et al. 2006). The white-skinned Pinot blanc is considered to have also arisen from Pinot noir. The insertion of a retrotransposon into one as well as the deletion of the other allele of the *VvmybA1* gene has blocked the production of anthocyanin in the white grape (Yakushiji et al. 2006).

The diversity of Pinot clones results not only in different color variants, but also in differences in cluster architecture, including loose or compact clusters, as well as in differences of the berry size. The overall number of clones of the Pinot family as well as the exact sources are still unknown to this day. Amongst several other mutational incidents, transpositional activities of retrotransposons are discussed to be a potential source for spontaneously occurring mutations leading to clonal variation in the grapevine genome (Forneck 2005).

In this study variation among Pinot noir clones was investigated, implementing the retrotransposon-based marker system S-SAP. The goal of this study was to target retrotransposon sequences within the grapevine genome. Additionally, the efficiency of the S-SAP marker system in the differentiation of closely related Pinot noir clones was examined. The S-SAP procedure was modified utilising universal retrotransposon primers instead of sequence-specific primers. By using universal primers it was expected to target a wider spectrum of retrotransposon sequences or remnants thereof in the grapevine genome.

II. Literature review

The following literature review outlines in more detail the significance of retrotransposons and their significance within genomes of woody perennials. In the beginning, the structural characteristics and the classification of retrotransposon families are presented, followed by a general overview of abundance and distribution in plant genomes. Moreover the applicability of retrotransposons as molecular tool is shown and illustrated on selected perennial fruit crops, showing similarities to grapevine such as vegetative propagation or extended periods of juvenility. Finally, the prevalence of retrotransposons in the grapevine genome as well as their influence on it is described.

Transposable elements, also known as jumping genes, are mobile DNA sequences found in most living organisms, prokaryotic as well as eukaryotic. They have the capability of jumping within the genome, and thus change their genomic location. Moreover they are able to amplify their copy number, increasing the genome size of their host. Their presence and activity can lead to major morphological and regulatory mutations (Capy et al. 1998). According to the mechanism of transposition, transposable elements are classified into two main groups, Class I and Class II elements. Class I elements or retrotransposons disperse in the genome via an RNA intermediate in a "copy and paste" mode. Class II elements, known as DNA transposons, propagate directly in a "cut and paste" fashion via a DNA intermediate (Bennetzen 2000).

DNA transposons were the first described transposable elements in plants. In the 1940s Barbara McClintock identified two transposons, named *Ac/Ds* (*Dissociator/Activator*) elements, in the maize (*Zea mays*) genome. She found that the transpositional activity of these elements affects the pigment synthesis in maize kernels, leading to variable coloration patterns (Capy et al. 1998).

II. 1. Retrotransposons

II. 1. 1. Structure and classification

Retrotransposons comprise retroviruses, long terminal repeat (LTR) and non-LTR retrotransposons, depending on their molecular structure and sequence similarities.

Retroviruses and LTR retrotransposons both share characteristic LTR sequences which flank the internal coding region of the elements. LTRs usually constitute a few hundred to several thousand base pairs, and contain regulatory sequences essential for the expression and integration of the retroelement (Suoniemi et al. 1997, Kalendar et al. 1999). The internal coding region includes two major genes, *gag* and *pol*. *Gag* encodes capsid proteins, responsible for the packaging of the RNA transcript, while *pol* encodes proteins essential for enzymatic activities. The *pol* domain comprises protease (prot), integrase (int), reverse transcriptase (RT), and RNAse H. Between the flanking LTRs and the internal coding regions, sequences, significant for reverse transcription of the retrotransposons RNA intermediate, are located: the primer binding site (PBS; between 5' LTR and internal domain) and the polypurine tract (PPT; between internal domain and 3' LTR) (fig.1; Capy et al. 1998). These key entities, PBS as well as PPT, appear to be well conserved within plant species and plant retrotransposon families (Suoniemi et al. 1997).

LTR retrotransposons can be further subdivided into Ty1-*copia* and Ty3-*gypsy* elements[1], according to the location of *int* domain within the *pol* gene (fig.1). Retroviruses show the same gene structure as Ty3-*gypsy* retrotransposons, except for an additional gene, *env*, which enables the package of the virus in an membranous envelope (fig.1). Thereby retroviruses represent the only infectious retroelements with the capability to spread from cell to cell as well as from organism to organism (Grandbastien 1992, Bennetzen 2000). Retroelements structurally resemble two elements which are both lacking the coding capacity and thus autonomous transposition as well: terminal-repeat retrotransposons in miniature (TRIMs; Witte et al. 2001) and large retrotransposon derivatives (LARDs; Kalendar et al. 2004). TRIMs contain short LTRs (100-250bp), PBS and PPT motifs but do not constitute any coding domain while

[1] Nomenclature of LTR retrotransposons was given according to the initial representatives first described for yeast (transposon yeast = *Ty*) and Drosophila (*copia/gypsy*).

LARDs show core domains but fail to encode protein products (Witte et al. 2001, Kalendar et al. 2004).

Non-LTR retrotransposons lack long terminal repeats but include a poly (A) track at the 3' end. They are subdivided into long interspersed nuclear elements (LINEs) and short interspersed elements (SINEs; (Kumar and Bennetzen 1999, Casacuberta and Santiago 2003). LINEs, several kilobases long, contain two open reading frames (ORFs). One encodes a capsid protein analog to the *gag* gene of retroelements, and the other encodes reverse transcriptase (RT), endonuclease (EN) and RNAse H (fig.1). The much smaller SINEs (100-500bp), in contrast, lack any coding capacity, and thus utilize *trans*-acting reverse transcriptase (Schmidt 1999).

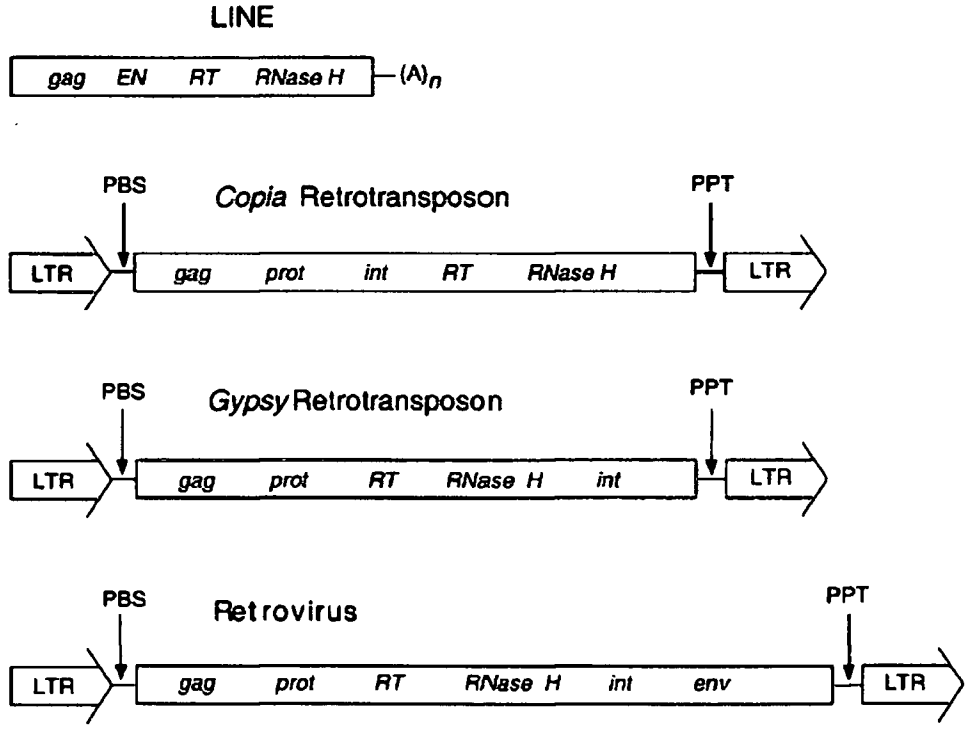

Figure 1: DNA structure of retroelements. LINEs are non-LTR retrotransposons, comprising a poly (A) track but lacking long terminal repeats (LTRs). LTR retrotransposons are divided into *copia* and *gypsy* elements, differing in the location of *int* domain. Retroviruses show the same structure as *gypsy* retrotransposons, but with the additional *env* gene. (Bennetzen 2000)

II. 1. 2. Abundance and distribution

Retrotransposons appear to be restricted mainly to eukaryotic organisms, different to DNA transposons which have been also revealed in most prokaryotes. LTR retrotransposons are found in most eukaryotes and can constitute large fractions of repetitive DNA, especially in plant genomes (e.g. Hirochika et al. 1992). In contrast, the presence of the structural similar retroviruses in plants is still not clarified. It is assumed, though, that their intercellular and interorganismal infectious nature also allows them to spread among plants and not only among animals (Peterson-Burch et al. 2000). Non-LTR retrotransposons have been discovered in plant genomes as well as in fungi, invertebrates, and mammals. SINEs, in particular, have been most extensively studied in the animal kingdom. In plants only few families have been identified, which probably reveals their limited significance in this kingdom (Fawcett et al. 2006).

Within plant genomes retrotransposons present a great variation in copy numbers: *Arabidopsis thaliana* is comprised of about 5% (Kapitonov and Jurka 1999), *Oryza sativa* of 10% (Mao et al. 2000), *Zea mays* of at least 50% (Bennetzen 2000), and within *Hordeum vulgare* they make up even 70% of the genome (Vicient et al. 1999).

The genome size and the copy number are positively correlated, indicating that retrotransposons have played an important role in the evolutionary history of plants (Bennetzen and Kellogg 1997). Their long term presence in plant genomes supports the theory of coevolution and coadaptation (Kidwell and Lisch 1997).

Coadaptation has probably reduced negative effects on the host and even lead to beneficial host functions useful for evolution. Depending on the involved host DNA, retrotransposons have generated various modifications: Retrotransposon insertions into coding regions, involving regulatory and exonic sequences, have caused changes in the expression of genes and thus often lead to phenotypic variability (Kidwell and Lisch 1997, Bennetzen 2000). Sequence analyses of promoters have even indicated a potential derivation from fragments of retrotransposons (Wessler et al. 1995). Insertions into non-coding regions, close to genic areas, may have also lead to modifications due to transcriptionally active sequences in LTRs (Casacuberta and Santiago 2003).

Retrotransposons often show a specific target site preference within the genome. Heterochromatin, characterised by repetitive sequences, is a preferred target region and considered to be "safe havens" for elements (Kidwell and Lisch 1997). The probability

of elemination by host mechanisms is mitigated whereby they can contribute to the genome size. In general, heterochromatin mainly constitutes inactive retroelements without having any impact on the host genome. Most retrotransposons, in fact, have lost their transpositional capacity during evolution. Defective elements even outnumber functional elements significantly, by several thousand copies (Weising et al. 2005). This is particularly evidenced for LTR retrotransposons (Casacuberta and Santiago 2003). Further landing pads are provided by retrotransposons themselves. By inserting within each other they build nested clusters referred to as Intergene LTR retrotransposons (IRPs; Bennetzen 2000). IRPs can make up large genomic fractions such as in maize, where they comprise up to 70% of the whole genome (SanMiguel et al. 1996). Besides these complex compositions retrotransposons are also present partially. Recombination between LTRs of single elements can produce solo LTRs by eliminating the internal coding region (SanMiguel et al. 1996, Vicient et al. 1999). In general, target site preferance for genetically inactive regions has prevented host genomes from several mutations.

Due to their insertional nature and high distribution within some plant genomes, retrotransposons provide an excellent basis for developing molecular marker systems. In the following chapter the main retrotransposon-based systems in use are presented.

II. 2. Retrotransposon-based molecular marker systems

Retrotransposon-based marker technologies are PCR-based multilocus systems which reveal polymorphism relying on the transpositional activity of retrotransposons. In plant genomes they are applied for cultivar identification, phylogenetics, and the construction of linkage maps for marker assisted breeding (MAS) and map-based cloning of genes (Kumar and Hirochika 2001).

Waugh et al. (1997) introduced a fingerprinting technique, known as sequence-specific amplified polymorphism (S-SAP), which is based on the basic principles of amplified fragment length polymorphism (AFLP; Vos et al. 1995). The original S-SAP procedure, conducted on the barley genome (*Hordeum vulgare*), amplified fragments containing a *Bare-1*-like retrotransposon LTR primer and a adaptor-homologous AFLP primer. No produts were gained using single *Bare-1* LTR, suggesting that *Bare-1* elements are not likely existent in an opposite orientation or not sufficiently close to each other for successful amplification. The overall proportion of polymorphic markers, generated by the *Bare-1*-driven S-SAP in combination with AFLPs , among two barley lines was high (Waugh et al. 1997).

Further retrotransposon-based fingerprinting techniques, first also described on the basis of *Bare-1* retrotransposon in *Hordeum*, are Inter-Retrotransposon Amplified Polymorphism (IRAP) and Retrotransposon-Microsatellite Amplified Polymorphism (REMAP; Kalender et al. 1999). In IRAP outward-facing LTR primers are utilised producing amplification fragments between two LTRs. In REMAP the LTR primers are additionally combined with simple sequence repeat primers (SSRs). In contrast to S-SAP, both approaches do not require DNA digestion by restriction enzymes. Moreover it has been possible to generate bands from a single LTR primer (IRAP) unlike in S-SAP. IRAP bands indicate a potential opposite orientation of *Bare-1* elements (Kalendar et al. 1997, Waugh et al. 1997). IRAP as well as REMAP amplified complex banding patterns between *Hordeum* species reflecting a high feasibility for the generation of markers (Kalendar et al. 1997).

Flavell et al. (1998) implemented a co-dominant, retrotransposon-based marker system in pea (*Pisum sativum*), known as Retrotransposon-based Insertion Polymorphisms (RBIP). The used primers derive from LTR retrotransposons and their host insertion site sequences, and thus require sufficient sequence information in advance. Insertion site sequence information can be gained by sequenced polymorphic

S-SAP bands (Flavell et al. 1998, Pelsy et al. 2003). The original RBIP, based on a *PDR1* LTR retrotransposon and the flanking host DNA, provided highly polymorphic single loci in pea and reflected to be an efficient system for high throughput analyses (Flavell et al. 1998).

A main shortcoming of these retrotransposon-based marker systems is the acquirement of sufficient sequence information to design retrotransposon-specific primers. Thus, it is necessary to clone and sequence several sequences in advance to gain an appropriate terminal sequence as primer. For the rapid isolation of LTR sequences of plant *Ty1-copia* retrotransposons a PCR-based method was introduced by Pearce et al. (1999). This approach can be potentially utilised for any plant species (Pearce et al. 1999). In comparison to other PCR-based multilocus marker systems, retrotransposon-based ones have mostly revealed higher levels of polymorphism (Waugh et al. 1997, Kalendar et al. 1999, Bretó et al. 2001). Amongst others, S-SAPs have been more polymorphic than AFLPs (Waugh et al. 1997, Bretó et al. 2001), and also IRAPs have exceeded the degree of polymorphism of ISSRs (Kalendar et al. 1999, Bretó et al. 2001), RAPDs, and SSRs (Bretó et al. 2001). The high proportion of polymorphic bands, emerging from insertional polymorphism, makes them to be less labour-intensive and more efficient approaches for developing markers.

II. 3. Distribution in woody plants and retrotransposon-based markers

Citrus is a perennial fruit crop which is vegetatively propagated via cuttings. As grapevine it shows a tendency to spontaneously occurring mutations affecting horticultural traits such as fruit characteristics. Retrotranspositional activity, involving homologous recombination and insertions, has been also discussed as potential source for somatic mutations in C*itrus*.

The presence of *copia*- and *gypsy*- retrotransposons has been investigated by several research groups. Asins et al. (1999) studied the occurrence of *copia*-like retrotransposons throughout the *Citrus* genome. By using a PCR assay designed to detect *copia*-like reverse transcriptase domains, most highly conserved retrotransposon sequences, they revealed numerous copies of *copia*-like elements within C*itrus ssp.* (Asins et al. 1999). Bernet and Asins (2004) observed the presence and genomic distribution of *gypsy*-like retrotransposons in the genome of *Citrus* and *Poncirus*. They isolated sequences comprising partial *pol* regions homologous to diverse plant *Ty3-gypsy* retrotransposons. Half of them were considered to be still potentially active elements, lacking inframe stop codons as well as frame-shift mutations. Further southern hybridization analyses and mapping of *gypsy* based IRAPs showed a nested pattern of the elements (Bernet and Asins 2003). Moreover they found high homologies to clustered *gypsy*-like domains located between resistance genes for *Citrus Tristeza Virus* (*CTV*) in *Poncirus trifoliata*, the only citrus species unsusceptible for this virus pathogen (Bernet and Asins 2003, Yang et al. 2003). In general, retrotransposons existing in resistance-gene clusters appear to be particularly involved in variability, resulting in novel resistance phenotypes. A recent study, conducted on *Citrus sinensis*, shows that 23% of the genome contains LTR retrotransposons: *copia*-representatives comprise 13% while *gypsy*-elements make up 10% (Rico-Cabanas and Martínez-Izquierdo 2007). Additionally, they found seven new *copia*-like retrotransposon families, classified according to their *reverse transcriptase* similarities. One of them was completely isolated and named *CIRE1*, representing the first complete retrotransposon described in *Citrus* species. By measuring *CIRE1* transcripts level, they proved the remained transcriptional activity of the element in specific plant tissue (Rico-Cabanas and Martínez-Izquierdo 2007).

For future studies, the detection of retrotransposons supports the isolation of new molecular markers for genetic studies concerning variability and disease resistance in *Citrus* (Bernet and Asins 2003). So far, IRAPs, based on *gypsy*- as well as on *copia*-elements, have been designed and successfully used for identification as well as distinction of cultivars (Asins 1999, Bretó et al. 2001, Bernet et al 2003).

Malus clones ("sports") are also considered to have arisen from retrotranspositional activity causing bud mutations during vegetative propagation. Bud mutations in *Gala*, for instance, have affected the colour intensity of fruits leading to various clones such as *Royal Gala* or *Galaxy* (Venturi et al. 2006). Few retrotransposons in the apple genome have been described so far (Yao et al. 2000, Antonius-Klemola et al. 2006).

Yao et al. (2001) have identified a LTR retrotransposon, named *dem1*, in the *MADS*-box transcription factor of the *MdPI* gene, the key regulator for apetaly and parthenocarpy in apple mutants. The insertion of *dem1* has altered the expression of the gene leading to parthenocarpic fruit development. Sequence analyses identified several stop codons and frameshifts within the retrotransposon indicating the loss of activity (Yao et al. 2001). S-SAP studies, implementing LTR primers based on *dem1*, were successful in revealing a high degree of polymorphism in *Gala* clones (Venturi et al. 2006). Antonius-Klemola et al. (2006) have cloned three full-size TRIM elements, a sub-group of LTR retrotransposons, in the apple cultivar *Antonovka*. Their further application as molecular markers, as IRAPs and REMAPs, showed the general potential of retrotransposons for revealing polymorphism in apple. Both, the TRIM primers itself (IRAP) as well as combined with microsatellite primers (REMAP), generated multiple polymorphic banding patterns. In this study, however, a definite distinction of sport mutations was not possible (Antonius-Klemola et al. 2006). In fact, S-SAPs appear to represent the only marker system at the time, which has the capability to differentiate clones in apple (Venturi et al. 2006).

The oil palm, including the African (*Elaeis guineensis* Jacq.) and the Latin American (*Elaeis oleifera)* palm, is commercially used for oil production, gained from the seeds and the pulp of the fruits. Studies on retrotransposons have been mainly conducted in combination with studies on DNA methylation within the genome, considering a potentially correlation to somaclonal variation in oil palm (Jaligot et al. 2000, Price et al. 2002, Kubis et al. 2003).

Price et al. (2002) isolated multiple *copia*-like retrotransposons in *Elaeis guineensis* by revealing heterogeneous *reverse trancriptase* sequences. On the basis of bootstrapping the elements were divided into three classes with different abundance among the two oil palm species. The methylation status of the retrotransposon classes was further investigated, showing that the ones occurring in high copy numbers tend to be more methylated, resulting to be inactive. A reduction of methylation, caused by abiotic and biotic stress, is correlated with the activation of retrotransposons. Functional retrotransposons may further generate somaclonal variation (Miura et al. 2001). In oil palm it has been shown that hypomethylation, occurring during clonal propagation through tissue cultur, leads to "mantling", a flowering abnormality (Jaligot et al. 2000, Kubis et al. 2003). Kubis et al. (2003), thus, investigated the DNA methylation as well as the distribution and content of different retrotransposon families in affected and normal plants. By using *reverse transcriptase*-based primers, *LINEs* and *gypsy*-like element sequences were cloned. Sequence analyses revealed the presence of *LINEs* in low copy number while *gypsy*-like retrotransposons showed a medium to high abundance within both genomes. The methylation level positively correlated with the copy number: the higher the degree the higher was the copy number of the retroelement. A strong reduction of DNA methylation was observed during tissue culture, which is considered as stress condition. The mantled plants showed minor methylation levels than the unaffected ones. However, a correlation between retrotranspositional activity and the mantling phenotype, which results in fruit abortion, was not confirmed (Kubis et al. 2003).

Cashew (*Anacardium occidentale* L.) is economically cultivated for the production of nuts. The development of molecular markers to aid breeding efforts is required since the crop shows a long period of juvenility, lasting between 3-5 years. Syed et al. (2005) developed S-SAP marker systems based on LTR sequences, which were gained by preceding isolation: two *copia*-LTRs, named *Tao1* and *Tao2*, were

isolated by PCR assays involving conserved regions in the *RNAseH* gene. Comparison of the retrotransposon-based marker system to AFLP showed that both revealed a comparable number of bands while SSAPs appeared to be more polymorphic within the cashew genome. Thus, they demonstrated to be efficient in revealing high polymorphism which may be used for future marker-assisted selection (Syed et al. 2005).

II. 4. Distribution in grapevine and retrotransposon-based markers

RAPD amplification products have provided the first indication for the presence of retroelements and remnants thereof in the grapevine genome. Cloned repetitive sequences showed high similarities to retrotransposons of higher plants, and found to be dispersed throughout the genome (Böhm and Zyprian 1998).

So far, 3 retrotransposons have been characterised. Verriès et al. (2000) have identified a truncated LTR retrotransposon, known as *Vine-1*, in the cultivar *Danuta*. The 2396 bp-long *Ty1-copia*-like element is located in the coding region of the *Adhr* gene, but has generally appeared to be present in multiple copies within the grapevine genome (Verriès et al. 2000, Labra et al. 2004). Pelsey et al. 2002 reconstructed another family of *Ty1-copia*-like retrotransposons, named *Tvv1*. *Tvv1* elements size between 4970 to 5343 bp and possess a single full-length ORF. Hypothetical translation of some representatives revealed stop codons or frameshifts indicating an inactivity of these retrotranspsosons (Pelsy and Merdinoglu 2002). Kobayashi et al. (2004) found a *Ty3-gypsy*-like retrotransposon, *Gret1*, which is dispersed throughout the grapevine genome. The 8774 bp-long element is associated with the mutation in the *Myb* related gene *VvmybA1* which regulates the anthocyanin biosynthesis in grapes. The insertion of *Gret1* in the 5'-flanking region near the coding sequence of the *VvmybA1* appears to have originally blocked the production of anthocyanin in dark-skinned grapes leading to white-skinned cultivars (Kobayashi et al. 2004). Recent studies on the skin colour mutation of grapevine have been conducted on the black-skinned Pinot Noir and the white-skinned Pinot Blanc (Yakushiji et al. 2006). Pinot noir, heterozygous for *VvmybA1*, comprises a functional allele, capable of anthocyanin expression, and a non-functional allele, which has lost its capability by the insertion of *Gret1*. Whereas Pinot blanc, considered to be arisen from the dark-skinned Pinot noir, possesses only a non-functional allele lacking the functional part of the gene. Studies on the identification and

isolation of this null-allele in Pinot blanc are in progress (Yakushiji et al. 2006). Moreover it has been observed that recombination between the LTRs of *Gret1* have lead to solo LTRs or even to a total loss of the retrotransposon in coloured cultivars. This excision event resulted in new dark-skinned varieties originated from white progenitors, such as Red Chardonnay which is derived from Chardonnay (Kobayashi et al. 2004, Yakushiji et al. 2006).

Besides these three characterised elements (*Vine-1, Tvv1, and Gret1)* multiple, still unknown retrotransposons are expected within the grapevine genome (Benjak et al. in prep.).

Retrotransposons have repeatedly been used for studying polymorphisms among grapevine cultivars and clones, and have revealed promising results. Relying on the presence of retrotransposon reverse transcriptase sequences, inverse sequence-tagged repeat analyses (ISTR) have been conducted for investigating genetic diversity among closely related *Sangiovese* accessions. ISTR fingerprints provided a high level of polymorphism whereby clonal distinction was successful (Sensi et al. 1996). S-SAP analyses, implementing primers based on the LTRs of *Vine-1*, were successful in distinguishing particular clones such as *Traminer* clones. But the distinction of *Pinot* clones failed, indicating different clonal variability in different cultivars (Imazio et al. 2002, Labra et al. 2003). Pereira et al. (2005) utilised molecular markers based on LTRs of *Gret1* for REMAP and IRAP analyses. In this present study, polymorphism among Portuguese cultivars was revealed leading to a successful identification, while the techniques failed in finding polymorphisms between clones of the same cultivars. Pelsey et al. (2003) have assessed the discriminative power of S-SAPs, relying on the LTRs of grapevine retrotransposons, within 12 *Vitis vinifera* varieties. They confirmed their efficiency in distinguishing each variety from one another (Pelsey et al. 2003).

These results indicate an efficient application of retrotransposon-based molecular markers for the identification and classification of grapevine cultivars and especially of closely related accessions.

III. Paper

Retrotransposon-based Variability in Pinot Noir Clones revealed by S-SAP

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III. 1. Abstract

Retrotransposons are ubiquitous in plant genomes. They possess the capability of changing their genomic locations and increase their copy number whereby they cause mutations. In this study we modified the S-SAP (sequence-specific amplified polymorphism) method using universal primers for retrotransposons in order to screen a wider range of elements. We were able to differentiate four out of five Pinot noir clones. Putative but still uncharacterized retrotransposons are contributing to clonal variation in grapevine clones.

Keywords: retrotransposons, S-SAP, clonal variation, Pinot noir

III. 2. Introduction

Propagated vegetatively, grapevine comprises a range of clones differing in minor genetic and phenotypic characteristics. Clonal variability ensures adaptation to new environmental conditions and is induced by various mechanisms. One explanation for variation within the progeny is the occurrence of spontaneous mutations (Forneck 2005). Potential sources for mutations are transposable elements, which possess the capability of changing their genomic location. Thereby they can alter gene structure and rearrange whole genomes causing major mutational changes (Kidwell and Lisch 1997, Bennetzen 2000). Class I elements, or retrotransposons, transpose via an RNA intermediate, which is reverse-transcribed into cDNA prior insertion into a new target location, whereas Class II elements, or DNA transposons, transpose directly via a DNA intermediate.

Retrotransposons are divided into long terminal repeat (LTR) and non-LTR and further into families according to their molecular structures and sequence similarities. LTR retrotransposons are composed of LTRs at both ends flanking the internal coding region which is characterized by two major genes, *gag* and *pol*. *Gag* encodes for a capsid protein while *pol* encodes for RNAse H, reverse transcriptase, protease and integrase, all needed for enzymatic activities (Casacuberta and Santiago 2003). Reverse transcription of the RNA intermediate of a retrotransposon starts at the 5' end of the internal domain, referred as the primer binding site or PBS (Capy et al. 1998), which is a potential target sequence for investigation and detection of retrotransposons by PCR amplifications.

The copy number, the abundance and the insertion sites of retrotransposons within most investigated plant genomes are considered to be a promising basis for the development of genetic marker systems (Kumar and Hirochika 2001). Several retrotransposon-based marker technologies have been developed in order to detect a higher degree of polymorphisms at the DNA level. Waugh et al. (1997) established a fingerprinting technique, known as sequence-specific amplified polymorphism (S-SAP). By using sequence-specific retrotransposon primers in combination with AFLP adapter primers a high level of polymorphism is revealed. Kalendar et al. (1999) introduced two retrotransposon-based fingerprinting techniques, Inter-Retrotransposon Amplified Polymorphism (IRAP) and Retrotransposon- Microsatellite Amplified Polymorphism (REMAP), both relying on the positions of LTRs of retrotransposons in the genome.

Outward-facing primers, binding to a LTR, are used for the IRAP method, and anneal between two LTRs while primers for REMAP anneal between LTRs and simple sequence repeats. Flavell et al. (1998) introduced a co-dominant marker system based on insertional activities of retrotransposons, known as retrotransposon-based insertion polymorphism (RBIP). The inverse sequence-tagged repeat analysis (ISTR), first implemented by Rohde (1996), relies on the presence of reverse transcriptase sequences of retrotransposons, and has already been used as molecular tool for investigating genetic diversity among closely related grapevine clones (Sensi et al. 1996).

In general, investigations on clonal variation within grapevine cultivars have shown that the degree of detected genetic divergence usually depends on the applied marker system and on the scope and type of plant samples (Forneck 2005). The retrotransposon-based marker systems SSAP or ISTR have shown higher levels of polymorphism (Labra et al. 2004, Sensi et al. 1996) than the standard AFLP. Clonal variation studies conducted on Pinot clones often failed but were successful in other clones, such as Traminer, indicating different clonal variability in different cultivars (Sensi et al. 1996, Regner et al. 2000, Imazio et al. 2002).The main disadvantage of the mentioned approaches is the need of adequate sequence information for specific primer design. The following study presents a modified transposon display approach based on the original S-SAP procedure (Waugh et al. 1997). The modification implies alternative primers which are universal for various plant retrotransposons. Thus, specific sequence information is not required. By applying universal primers we expect to target multiple retrotransposon sequences in the grapevine genome. It is entirely possible to detect an even wider spectrum of mobile elements including all retroelement related sequences which have affected the genome for thousands and millions of years. These elements might have survived within intergenic areas or even within coding and regulatory regions such as promoters (Kidwell and Lisch 1997). In fact, many promoters of plant gene sequences contain fragments of transposable elements, indicating a possible contribution to their origin (Wessler et al. 1995, Bennetzen 2000). By targeting a wide range of unknown retrotransposon sequences the chance of detecting elements, which may have caused changes of evolutionary significance for the grapevine genome, increases. These retrotransposon induced mutations may be also responsible for clonal variation among Pinot noir, resulting in genetic and even phenotypic differences.

In this study, we wanted to test the efficiency of a modified S-SAP approach for revealing polymorphism based on retrotransposon sequences. At the same time we

tested the method's capability of differentiating between Pinot noir clones, which are difficult to distinguish genetically.

III. 3. Material and methods

III. 3. 1. Plant materials

Six *Vitis vinifera* samples were used for analysis: five clones (20Gm, 1-44Gm, 18Gm, 20-13Gm, 1-84Gm) of the cv. Pinot noir (Forschungsanstalt Geisenheim, Germany) and the clone ST49 of the cv. Domina (Nursery Steinmann, Sommerhausen/Main, Germany). The Domina clone 'ST49' is a young crossing between Pinot noir and Portugieser, and was used here as outgroup. To check the reproducibility of the method repetitions of all 6 samples were included from the beginning, resulting in a set of 12 samples. Genomic DNA was extracted from fresh leaves using E.Z.N.A. SP Plant DNA Miniprep Kit according to the manufactorer's instructions (Omega Bio-tek, Doraville, USA).

III. 3. 2. Transposon display

Extracted DNA (13.5 µl) was restricted with *Mse*I (Fermentas GmbH, St. Leon-Rot, Germany) in a total volume of 25µl. The digestion was conducted for 2 h at 65°C. Restricted DNA was further purified using Perfectprep® Gel Cleanup Kit (Eppendorf, Hamburg, Germany). After purification template DNA (25 µl) was prepared by adding 5 µl of a ligation mix (50 pmol *Mse*I adapter, 100 mM ATP, 10x T4 Ligase buffer and 1 U T4 Ligase (Fermentas GmbH), and was incubated over night at room temperature (20°C). T4 Ligase was inactivated by heating up to 65°C for 10 min, and samples were stored at 4°C. In the preamplification step the primer M (0) (tab.1), homologous to the adapter sequence, was combined with 6 labeled (IRD700 and IRD800) universal retrotransposon primers: F0100, F0103, F0104, F0105, F0113, F0117 (Kalendar, personal communication).

The PCR reaction mixture contained 2.25 µl template DNA, 1.5 µM M(0), 1.5 µM transposon primer, 1x PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs and 1 U Taq DNA polymerase recombinant (Invitrogen Ltd., Paisley, UK) in a final volume of 15 µl. The unselective PCR was conducted using the following program: 94°C · 60 s^{-1} + 26 x (94°C· 30 s^{-1} , 56°C · 60 s^{-1}, 72°C · 60 s^{-1}) + 72°C · 6 min^{-1}. The preamplified DNA

was diluted (1:10) and stored at 4°C. The selective amplification was carried out in a total volume of 10µl containing 1 µl of preamplified DNA, 0.5 µM selective MseI primer (M22, M23, M24, M25, M27) (tab.1), 0.5 µM transposon primer, 1x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.75 U Taq DNA polymerase recombinant (Invitrogen) using the following cycle profile: 94°C · 60 s^{-1} + 12 x
(94°C· 30 s^{-1}, 65°C · 30 s^{-1}, 72°C · 60 s^{-1}) [The annealing temperature was reduced by 0.7°C in each of the 12 cycles.] + 26 x (94°C· 30 s^{-1}, 56°C · 30 s^{-1}, 72°C · 60 s^{-1}) + 72°C · 6 min^{-1}.
Bands were separated in a 8% polyacrylamid gel, and visualized by the automated LI-COR NEN 4300 DNA analyzer (Licor Biosciences GmbH, Bad Homburg, Germany).

Table 1: List of primers used in the PCR analyses.

Primer Code	DNA sequence
M(0)	5'- GAT GAG TCC TGA GTA A -3'
M22	5'- GAT GAG TCC TGA GTA ACA A -3'
M23	5'- GAT GAG TCC TGA GTA ACT T -3'
M24	5'- GAT GAG TCC TGA GTA ACA C -3'
M25	5'- GAT GAG TCC TGA GTA ACA T -3'
M27	5'- GAT GAG TCC TGA GTA ACT G -3'

III. 3. 3. Sequencing

For further sequence analysis four polymorphic bands (tab.2) were cut out from the gels using the Odyssey Infrared Imaging system (Licor Biosciences GmbH), and stored in 20µl 1xTE buffer at 4°C. By three repeated steps of freezing (20 min) and thawing, the DNA has been dissolved from the polyacrylamid gel into the buffer. 1µl of each sample was used for a nested PCR, using M (0) and unlabeled transposon primers, to amplify the extracted band. The amplification was conducted using the selective PCR program described above in a total volume of 50µl. 40µl of the PCR products were resolved on a 1,5% agarose (1xTAE) gel and stained with ROTI-methylene blue staining concentrate (ROTH, Karlsruhe, Germany). The bands, including one repetition for each band, were cut out from the gel, purified with Perfectprep® Gel Cleanup Kit (Eppendorf) and sequenced.

The sequences were aligned in BioEdit software (Hall 1999), and compared against the nucleotide and protein databases at NCBI using BLAST (Alstschul et al.

1990) as well as against the Repbase Update database of transposable elements using the Repeat Masking tool (Jurka et al. 2005).

III. 3. 4. Statistical analyses

The bands were manually scored as present (+) or absent (-). Only reproducible as well as clearly visible bands were recorded. Similarity data matrices were calculated using SM coefficient in the NT-SYS PC program, Version 2.01 (Rohlf 1998). A dendrogram was constructed by SAHN using UPGMA method.

III. 4. Results

The transposon display method generated variable banding patterns among the samples (fig.2). Six different universal transposon primers (F0100, F0103, F0104, F0105, F0113, F0117) combined with five selective *Mse*I primers (M22, M23, M24, M25, M27) generated a set of 30 primer combinations. The amplified fragments varied in size between 50 bp and 350 bp. To check the reproducibility of the method, repetitions were included from the beginning. In general, the bands were reproducible except for few which were omitted from calculations to ensure consistency. The Domina samples showed different banding patterns than the Pinot samples: Bands were too weak or even missing in the upper molecular weight range in some primer combinations. In these situations bands from Domina as well as from Pinot were not recorded for statistical analyses (11%).

The study involved 5 Pinot noir clones varying in their cluster architecture (compact/ loose/ small berry) and one Domina clone, a young crossing between Pinot noir and Portugieser, which served as outgroup. Altogether 670 markers were generated by 30 primer combinations revealing 8,8% polymorphism (59 polymorphic markers in a total of 670 markers). Excluding polymorphic markers for the outgroup resulted in 4,8% polymorphism among the Pinot noir clones (tab.3). Based on the presence or absence of amplified fragments, a genetic similarity matrix was calculated using the SM coefficient and an UPGMA based dendrogram was created (fig.3). As expected, Domina could be outgrouped. Clones 20 Gm and 20-13 Gm were genetically indistinguishable, while the others could be distinguished. The overall similarity among the Pinot clones was generally high (97,5%), as expected with closely related accessions.

To further elucidate wether the amplified DNA fragments really derived from retrotransposon templates, four polymorphic bands, including repetitions, were randomly chosen and sequenced. Sequences from repetitions for each band were identical. The length of the sequenced fragments varied between 160 and 245 bp (tab.2). Sequence analyses confirmed full specificity to the outward-facing retrotransposon primers; i.e. all four sequences derived from correct primer annealing and were not artifacts from unspecific amplifications. Because the primers are designed as outward facing from the retrotransposon PBS region, our sequences should be theoretically parts of LTRs. The sequences do not match to any known retrotransposon in grapevine. Sequence analyses using BLASTn and tBLASTX algorithms against the database of Repbase Update (Jurka et al. 2005) and NCBI (Alstschul et al. 1990) did not confirm any matches to retrotransposons. This was expected, as LTRs are normally less conserved between different retrotransposon families and plant species. As confirmation, we blasted the LTR sequence of the *Gret1* retrotransposon from grapevine (accession number: AB242301) against the RepBase Update and NCBI database and found no additional hits.

LTR sequences usually start with TG and finish with CA motifs (Suoniemi et al. 1997), followed by 1-4 nucleotides before the conserved PBS region. All our sequences have the CA motif 2-4 nucleotides before the primer sequence (PBS) (tab.4).

Table 2: Four polymorphic markers of different clones and primer combinations were sequenced and used for sequence analyses.

Sequence	Clone	Primer combination	Sequence size in bp
1	1-44 Gm	F0117 - M24	245
2	1-44 Gm	F0104 - M24	220
3	18 Gm	F0104 - M27	228
4	18 Gm	F0103 - M25	160

Table 3: Detected polymorphism by the six universal primers. Total number of markers and polymorphic markers for Pinot/Domina clones.

Primer	Number of markers	Total polymorphic markers	Polymorphic markers for Pinot clones (%)	Polymorphic markers for Domina (outgroup)
F0100	174	20	13 (7,5 %)	7
F0103	132	10	5 (3,8 %)	5
F0104	171	15	6 (3,5 %)	9
F0105	69	4	2 (2,8 %)	2
F0113	54	4	2 (3,7 %)	2
F0117	70	6	4 (5,7 %)	2
Total	670	59	32 (4,8 %)	27

Table 4: Sequences of four polymorphic bands chosen for sequence analysis. *Mse*I primer sequence are single underlined (missing in sequence 1); PBS region (universal transposon primer sequence) double underlined; CA motif, 1-4 bases, before the PBS region in grey.

Sequence 1:
TGAAATAGCTATGATGCTCCCAAAACTCCTGTGGAGTGCGTGGTGTGGAT
CTTCAAAGYGGCAGCCCCCTTTCCAAAAATTCCATGCCTGTAAGTATCTCT
CCCATCTACAACCCTAAAGCATTCTCCAGCACAGTTTGCTTCATACCGGAA
TCATAGAACTGTGAGWTCATGTTGTCATCAACTGAGACCTTCTCATGAACC
GAGAGGCACGGGGWCCACAAAATGGCGACTCCGCTGGGGAT

Sequence 2:
GATGAGTCCTGAGTAACACAACCCATTGCATCTAGTGCCAGTCGTTGTTTT
AATGTATGGTGCCCGTCTCTTGTTGCTTGTTGGCTCAAAAGTAGAAATAATT
ANATTTGACTCCTAGTACTATAACTTTTCCACCTTTGAAAAGGCCCCATGAT
GTCTCTCCTAAATGCCTAATAAGTTAGAACATCCACATGGTATCAGAGCCC
CCTTGACCCTAG

Sequence 3:
GATGAGTCCTGAGTAACTGTTTGGAGATGGCTTGGTCCAGAAAATGCCAAA
GTGCAGTCATGGGTGTTTTCATATGGCGAATGAGGGTTTGGTACAAGCCTT
GCAAGCATTATAGCAGTCAGATTGATAACCCCAAGGTAGTGATCTCGGTAT
TGGGCACGAGCATTAGAAACAGTCCAGATCAAGTCTCACCCGTGGTATCA
GAGCCCCCTTGACCCTG

Sequence 4:
GATGAGTCCTGAGGTAACATGGCCGCGTGTTCTTCAAACCGGTATGTAATC
AATTCGTTAAATTTTTGAGATGTGTTGAATTCAATGATCTTGAATTTGTGTGT
TAATTTTCRCGTTAAATGCTAACAATTGGTATCAGAGCTCAAGTTGCTCGGT

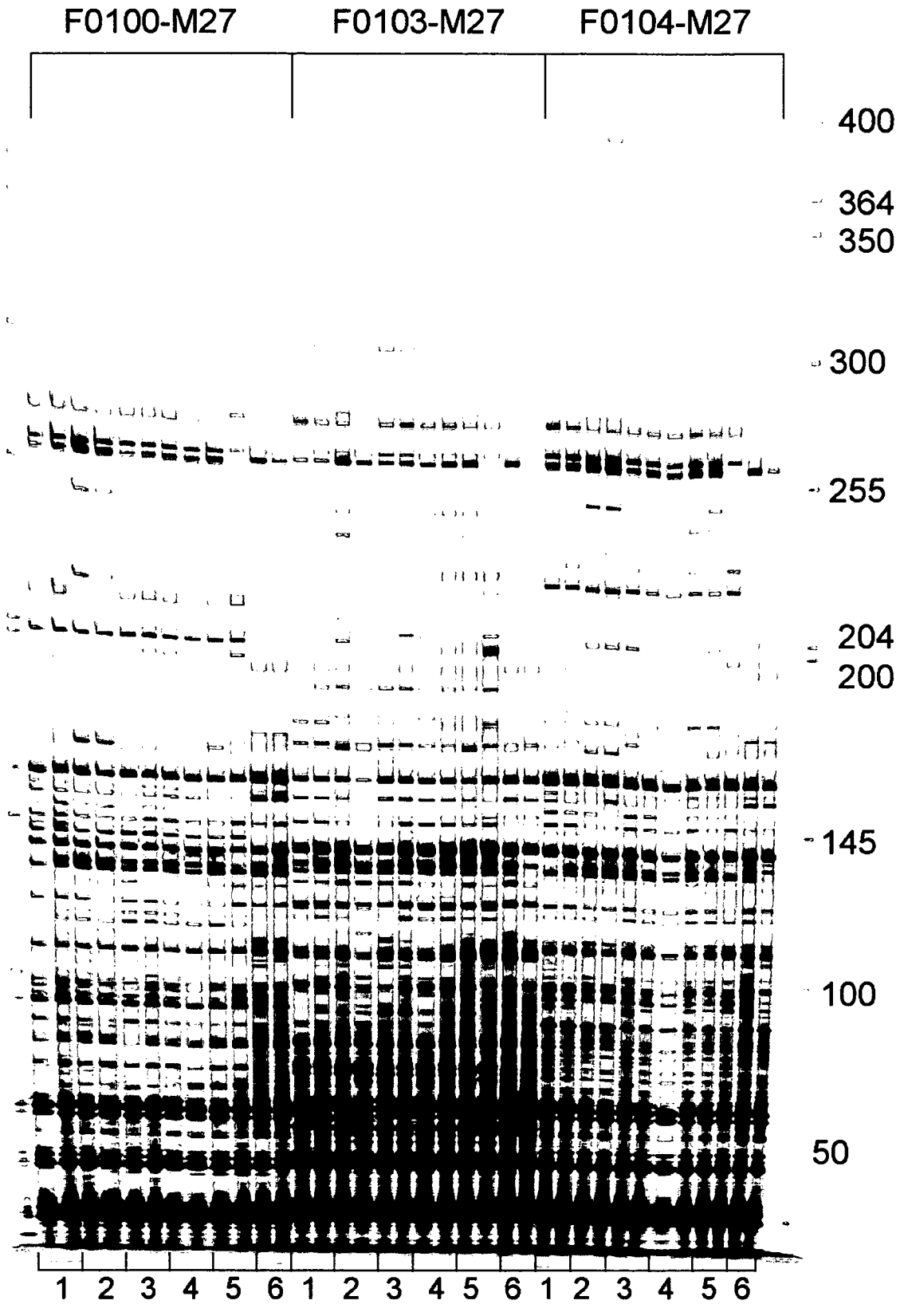

Figure 2: Gel including 3 primer combinations (F0100-M27, F0103-M27, and F0104-M27). Number 1-6 is the order of clones (in repetitions): 1=20Gm, 2=1-44Gm, 3=18Gm, 4=20-13Gm, 5=1-84Gm, 6=Domina (ST49).

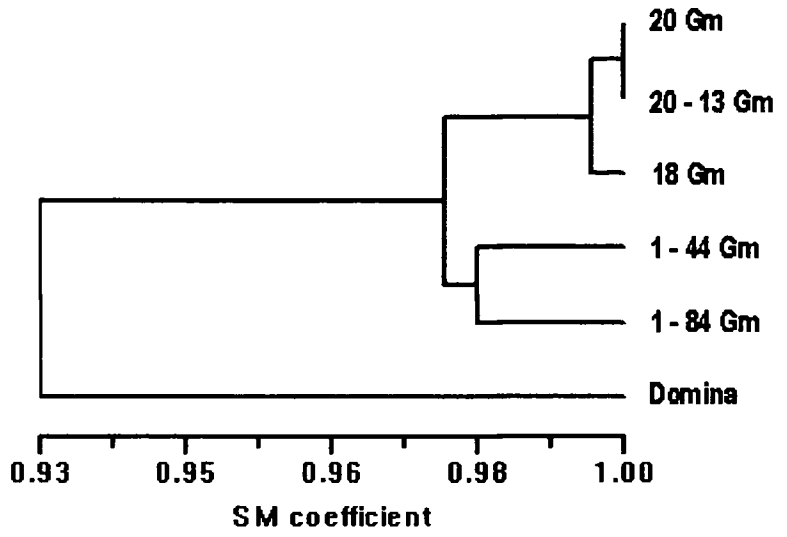

Figure 3: UPGMA based dendrogram (using the SM index) describing the genetic relationship among five Pinot noir and one Domina clones, obtained by a retrotransposon display method.

III. 5. Discussion

Retrotransposons are characterized by widespread dispersion and various copy numbers within plant genomes. Small genomes, such as *Arabidopsis thaliana* (125 Mb), comprise about 4-8%, while large genomes, such as *Hordeum vulgare* (5000 Mb), host a proportion of approximately 50-80% retrotransposons (Kumar and Bennetzen 1999). The abundance of retrotransposons in *Vitis vinifera* L. is yet to be determined. Regardless of the actual abundance, there seem to be multiple types of uncharacterized retrotransposons in grapevine. According to findings of Benjak et al. (in prep.) the grapevine genome comprises multiple unknown retrotransposons. So far, only three retrotransposons have been described: *Vine-1* (Verries et al. 2000), *Tvv1* (Pelsy and Merdinogluy 2002) and *Gret1* (Kobayashi et al. 2004).

For targeting retrotransposons we used universal primers, which anneal to conserved PBS of various plant retrotransposons, in this study. Confirming that these sequences match to LTR regions of retrotransposons we conducted BLAST analysis to databases of existing retrotransposons. No similarity matches were found, probably for two main reasons: First, all four sequences were short in length, varying between 160 and 245 bp and presenting only a small area of LTRs, which decreases the chance of revealing similarities with known LTRs. Second, the amplified LTR regions are less

conserved among families of retrotransposons, and thus comparison to existing databases is not reliable. The terminal LTR motif CA, characteristic for most retrotransposons and retroviruses (Suoniemi et al. 1997), gives the only evidence of dealing with LTR sequences. These LTR sequences may be flanking the internal coding regions of still unknown retrotransposons within the grapevine genome. The transpositional capacity of LTR retrotransposons has probably induced various mutations in the plant's evolutionary history. Kobayashi et al. (2004) has revealed a retrotransposon-induced mutation affecting the anthocyanin biosynthesis of red grapevine cultivars: The insertion of the retrotransposon *Gret1* into the 5'-flanking region of the *VvmybA1a* allele blocks its expression and thus the production of anthocyanin in grapevine berries. From the evolutionary point of view, it is assumed that the consequence of the *Gret1* insertion has been the origin of white cultivars. The high copy number of LTR-retrotransposons in plant genomes (Kumar and Bennetzen 1999) supports their potential contribution to still undiscovered mutational events, which may have lead to polymorphism among closely related grapevine accessions. In this study, implementing the S-SAP method, 8,8% polymorphism was revealed in total. The highest rate of polymorphism was obtained by the primer combination F0100 with all five *Mse* primers (20 out of 174 markers). Generally, the primers F0100, F0103 and F0104 generated significantly more polymorphic markers than FO105, FO113 and FO117 (data not shown). Differences in efficiency may occur since each primer was designed from a different group of plant retrotransposons (Kalendar, personal communication). Among the Pinot noir clones 4,8% polymorphism was revealed, suggesting a good usability of the modified S-SAP method for studying intraclonal variability in grapevine. Comparative studies between S-SAPs and other molecular markers, such as AFLPs or RAPDs, also supports the method's applicability by detecting higher levels of polymorphism than the others (Waugh et al. 1997, Berenyi et al 2002).

S-SAPs, implemented on Pinot blanc and Pinot gris clones, failed in revealing genetic variation (Labra et al. 2004). The differentiation of the Pinot noir clones, involved in this study, was successful: four out of five clones were distinguishable. The genetic indistinguishable clones represent subclone (20-13Gm) and clone (20Gm), reflecting a close genetic relationship.

This study shows again the useful applicability of retrotransposon- based marker systems in studying plant genomes. Relying on the unique features of retrotransposons, they reveal sufficient polymorphism for differentiating even close related plant accessions such as grapevine clones. The potential LTRs, discovered by sequence analyses of polymorphic markers, unequivocally suggest the presence of more unknown retrotransposon sequences in the grapevine genome. During evolution these elements have been responsible for transpositional activities, which probably have been contributing to clonal variation. To understand their long term evolutionary significance for grapevine clones better, further analyses are required. By obtaining more data of existing grapevine retrotransposons, conclusions about genetic and phenotypic variation among clones will certainly increase.

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IV. Conclusive discussion

The applied transposon display method has included the basic molecular steps of the AFLP technology: extraction of genomic DNA, digestion of DNA with restriction enzymes, adapter ligation, preamplification, selective amplification and gel analysis (Vos et al. 1995). According to the S-SAP protocol only one adapter-homologous AFLP primer, instead of a pair, in combination with universal retrotransposon primers were used.

Preliminary, extracted DNA was separately restricted with *EcoRI*, a rare cutter recognising a 6-base sequence motif, as well as with *MseI*, a frequent cutter with a 4-base recognition site. In both amplification steps each corresponding unselective primer, E (0) or M (0), was combined with the same retrotransposon primers. The combinations with the *MseI* primer significantly revealed more efficient banding patterns, suggesting M (0) to be superior for forthcoming amplification analyses than E (0). To further reduce the complexity of the fingerprints selective *MseI* primers with three additional nucleotides were utilised in the second amplification. Each selective base reduces the number of amplified fragments by a factor of four (Vos et al. 1995, Waugh et al. 1997), allowing an efficient visualisation and a clearer scoring of bands as present or absent. The used retrotransposon primers were homologous to the PBS region of various plant retrotransposons, thus reflecting to be universal. They were labelled with either IRDye 700 or 800 for further visualisation on an automated two-infrared dye DNA analyser, the LI-COR NEN 4300. Electrophoresis conditions are shown in table 5.

Table 5: Electrophoresis Conditions for LI-COR Biosciences NEN Model 4300 DNA Analyser.

	Prerun	Run
Image Width	1028	1028
Scan Speed	2	2
Channels	700,800	700,800
Voltage	1500	1500
Current	40	40
Power	40	40
Enable Heater	Yes	Yes
Temperature	45	45
Time	00:15	04:30

In total, 30 primer combinations, 5 selective *MseI* primers combined with 6 universal retrotransposon primers, were used for amplification. The amplified banding patterns varied between the different combinations. While 3 retrotransposon primers (F0100, F0103, and F0104) produced variable and intense patterns, the other 3 revealed insufficient and light bands. In some cases, it was even impossible to score bands. To improve the variability and intensity of such bands, the concentration of PCR products, applying on the gel, was increased in the first place. Hardly any improvements were observed. Secondly, the concentration of template DNA for PCR analyses was increased by 0,5µl. Thereby marginal improvements were achieved, but generally the banding patterns did not enhance compared to F0100, F0103, and F0104. Since each retrotransposon primer was designed from different plant retrotransposons differences in the performance cannot be excluded (Kalendar, personal communication).

Throughout analyses the performance of Domina samples was insufficient. They showed weak or even missing bands, especially in the upper molecular weight range of some primer combinations. Causes for inadequate efficiency might concern the DNA quality or amplification steps. To exclude deficiencies in PCR both, unselective as well as selective amplification, were repeated but no improvements were obtained.

In total, 670 bands were generated, revealing 4,8 % polymorphism among the Pinot noir clones. To trace the origin of the amplified DNA fragments, four polymorphic bands were sequenced. The derivation from the PBS binding primer was confirmed while BLAST analyses did not reveal matches to any known retrotransposon. The lack of similarity has been expected since LTRs are less conserved among retrotransposon families (Kalendar et al. 1999). Implementing outward-facing primers as well as the presence of the conserved *CA* motif, characterising the end of LTRs, indicated to have target LTR sequences. The origin of the remaining 666 amplified fragments, however, is unknown. Four sequences were confirmed to derive from correct primer annealing but this may be not allocated to the rest. Theoretically, some fragments might have resulted from retrotransposon-retrotransposon primer combinations. Considering that retroelements show a bias for inserting within each other, building up complex nested arrangements, it is possible that two PBS regions are present in opposite direction to each other. Thus, they represent target sequences for primer annealing resulting in DNA fragments. The amplified products varied between 50 and 350 bp in length.

In general, plant LTRs range between few hundreds to several thousand bp: the length of the grapevine *Tvv1*-LTRs is approximately 150 bp (Pelsey et al. 2002). Thus, it is possible to have revealed whole LTR sequences or additional flanking regions of the host genome. Thereby revealed polymorphism may be based on insertional mutations caused by retrotranspositional activity. Further it is also conceivable to have targeted truncated elements or solo LTR sequences, still comprising the conserved PBS region.

By revealing polymorphism among the closely related Pinot noir clones the retrotransposon-based S-SAP method proofed to be a promising marker system for future studies in grapevine. Their application fields may include identification and characterisation of cultivars, phylogeny, as well as construction of linkage maps for marker-assisted breeding programs. The diversity of grapevine varieties, and especially of closely related and diverse accessions such as Pinot clones, has often leaded to misinterpretations and wrong identification. The long history of cultivation and global distribution into different geographical and climatic regions has resulted in a wide range of phenotypes, whereby it has become difficult to prevent identification errors. Different countries often used different names for the same cultivars. Thus, reliable identification methods, complementing the traditional ampelographic procedures, have been desired, not only by wineries but also by breeders. Winemakers are strongly interested in a quality control which guarantees the correct origin of grape-vines for cultivation. Molecular markers, in fact, are very encouraging for identification since they can be used independently from the developmental stages of the plant. Especially, in breeding this is a major advantage since they allow early selection for a trait. Like other perennial fruit crops, such as citrus or cashew, grapevine is characterised by a relatively long juvenile period before flowering. Thus, breeders need to wait until an evaluation of the fruit quality is possible, which is unequivocally cost-intense and time-consuming. A major goal for breeding is to find markers closely linked to agronomically relevant traits, concerning plant and fruit performance. Instead of selecting directly for a trait, markers, tagging a particular trait, are selected. Consequently genetic linkage maps are constructed supporting marker-assisted selection which helps to reduce expenses and improve the efficiency of the breeding process. Especially, selection for novel resistance phenotypes has been of interest for agriculture.

Since retrotransposons have the capability to generate high variability within genomes, they have been associated with the evolution of disease resistant plants. In fact, the existance of retrotransposons within resistance gene clusters have been already proofed in some cases (e.g. Yang et al. 2003).

Moreover their presence and activity within fruit crop genomes have shown to be correlated with several fruit characteristics (Yao et al. 2001, Kubis et al. 2003, Yakushiji et al. 2006). In grapevine, retrotransposons are seen to be responsible for various colour mutants. Theoretically, they might have played a significant role in generating different grape clusters (compact or loose) as well. In this study, five Pinot noir clones varying in their berry cluster were implemented. The limited number of plant samples certainly does not give any evidence if the cluster architecture is correlated to retrotranspositional activities. But a relatively high level of polymorphism, relying on retrotransposon sequences, was achieved, generally suggesting to be an efficient molecular marker system for grapevine clones. However, it will be interesting to use a wide range of clones, differing in the berry clusters, in future S-SAP analyses. Markers linked to this particular fruit trait might be found which would enable a selection for the desired cluster type in grapevine.

This study revealed the existence of several unknown retrotransposon sequences which might have had a major impact on the evolutionary history of grapevine. The more we get to know about the presence and activity of these elements the more efficient are the improvements that can be made concerning breeding and cultivation of this economically important crop.

V. German abstract

Die Rebsorte Pinot noir (*Vitis vinifera* L.) ist charakterisiert durch eine große klonale Variabilität, die durch spontan auftretende Mutationen entstanden ist. Ein möglicher Mechanismus der Mutationen im Genom auslöst, ist die Aktivität von Retrotransposone. Retrotransposone sind mobile genetische Elemente mit der Fähigkeit ihren Genort zu wechseln. Dadurch können sie sowohl die Struktur als auch die Funktion der Gene verändern. In dieser Arbeit wurde die genetische Diversität von fünf Pinot noir Klonen mittels einer S-SAP Analyse (Sequence-Specific Amplified Polymorphism) untersucht. Statt Sequenz-spezifischen Primern wurden allgemeine Transposon-Primer, die komplementär zur konservierten PBS (Primer Binding Site) Region unterschiedlicher LTR-Retrotransposone sind, verwendet. Ziel der Untersuchung war einerseits die Effizienz dieser DNS-Analyse zur Differenzierung von Rebklonen zu testen, andererseits unbekannte Retrotransposon-Sequenzen im Rebengenom zu finden.

Insgesamt wurden 30 Primer Kombinationen, sechs Retrotransposon-Primer kombiniert mit fünf selektiven *MseI* Primern, eingesetzt, die im Ganzen 670 Amplifikationsprodukte produzierten. Vier polymorphe Banden wurden sequenziert und mittels BLAST Analysen untersucht. Der Sequenzabgleich mit BLASTn und tBLASTX ergab keine Übereinstimmung mit bereits vorhandenen Retrotransposonen. Aufgrund des Basenmotifs CA, das charakteristisch für das Ende von LTRs (Long Terminal Repeats) ist und das in allen vier Sequenzen gefunden wurde, wird jedoch davon ausgegangen, dass es sich um Retrotransposon-Sequenzen handelt. Zwischen den Pinot noir Klonen wurden im Rahmen des durchgeführten Screenings maximal 4,8% polymorphe Marker entdeckt, wodurch eine genetische Unterscheidung von vier Klonen möglich war. Dieses Ergebnis zeigt die Eignung der S-SAP Analyse zur zukünftigen Gewinnung von molekularen Markern, die für die Identifizierung engverwandter Rebklone und generell in der Rebenzüchtung Verwendung finden können.

VI. References

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