



Raimundo Maximino Martins Diz

Licenciado em Biologia

Functional genomic analysis of heat stress in *Vitis vinifera*

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Helena Sofia de Campos Pereira,
Investigadora, Instituto Superior de Agronomia

Júri:

Presidente: Prof. Doutor(a) José Paulo Nunes de Sousa Sampaio

Orientadora: Prof. Doutor(a) Helena Sofia de Campos Pereira

Arguente: Prof. Doutor(a) Jörg Dieter Becker



FACULDADE DE
CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Janeiro de 2015

“We are just an advanced breed of monkeys on a minor planet of a very average star. But we can understand the Universe. That makes us something very special.”

— Stephen Hawking

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Acknowledgments

First and foremost I would like to express my gratefulness to my supervisor, Sofia Pereira. I am forever thankful for the guidance, the support and help in all stages of this thesis. The encouragement in the lab work and knowledge given during all this past year was invaluable. For that and much more, was a pleasure and a lifetime experience.

My sincere thanks go to all the members of the Departamento de Genética from I.S.A., for their always joyful spirit and predisposition to help in the innumerable cases during this last year. A special word of appreciation to the director and soul of this department, Wanda Viegas, for all knowledge and motivation shared.

Finally, I want to thank my family and friends, who helped and supported my work, outside the work environment. All the relaxation moments and motivation were essential to balance the road of emotions lived through the past year.

Abstract

Grapevine (*Vitis vinifera*) is one of most agro-economically important fruit crops worldwide, with a special relevance in Portugal where over 300 varieties are used for wine production. Due to global warming, temperature stress is currently a serious issue affecting crop production especially in temperate climates. Mobile genetic elements such as retrotransposons have been shown to be involved in environmental stress induced genetic and epigenetic modifications. In this study, sequences related to Grapevine Retrotransposon 1 (*Gret1*) were utilized to determine heat induced genomic and transcriptomic modifications in Touriga Nacional, a traditional Portuguese grapevine variety. For this purpose, growing canes were treated to 42 °C for four hours and leaf genomic DNA and RNA was utilized for various techniques to observe possible genomic alterations and variation in transcription levels of coding and non-coding sequences between non-treated plants and treated plants immediately after heat stress (HS-0 h) or after a 24 hour recovery period (HS-24 h). Heat stress was found to induce a significant decrease in *Gret1* related sequences in HS-24 h leaves, indicating an effect of heat stress on genomic structure. In order to identify putative heat induced DNA modifications, genome wide approaches such as Amplified Fragment Length Polymorphism were utilized. This resulted in the identification of a polymorphic DNA fragment in HS-0 h and HS-24 h leaves whose sequence mapped to a genomic region flanking a house keeping gene (NADH) that is represented in multiple copies in the *Vitis vinifera* genome. Heat stress was also found to affect the transcript levels of various non-coding and gene coding sequences. Accordingly, quantitative real time PCR results established that *Gret1* related sequences are up regulated immediately after heat stress whereas the level of transcript of genes involved in identification and repair of double strand breaks are significantly down regulated in HS-0 h plants. Taken together, the results of this work demonstrated heat stress affects both genomic integrity and transcription levels.

Key words: *Vitis vinifera*, heat stress, *Gret1*, genomic modifications, transcription.

Resumo

A videira (*Vitis vinifera*) é um das culturas de fruto com mais relevância económica a nível mundial, com especial relevância para Portugal, onde existem mais de 300 castas específicas para a produção de vinho. Devido ao aquecimento global, altas temperaturas têm provocado um grande défice na produção agrícola, com especial relevância para regiões em climas temperados. Elementos genéticos móveis, como os retrotransposões têm sido correlacionados a alterações genéticas e epigenéticas, provocadas por stresses ambientais. Neste estudo, foram utilizadas sequencias relacionadas com o Grapevine Retrotransposon 1 (*Gret1*) para determinar se o stress térmico provoca alterações a nível da estabilidade genómica e transcrição. Para este objetivo, estacas já desenvolvidas de Touriga Nacional, umas das mais tradicionais castas portuguesas, foram submetidas a um stress térmico durante 4 horas a 42 °C, para posterior análise a nível de transcrição e estabilidade genómica em regiões codificante e não-codificantes, utilizando para isso DNA e RNA extraído de folhas recolhidas logo a seguir ao stress térmico (HS-0 h) ou após um período de recuperação de 24 horas (HS-24 h). Foi demonstrado que o stress térmico leva a um decréscimo significativo de sequências relacionadas com o *Gret1* a HS-24 h, indicando assim, que este tipo de stress leva a alterações na estrutura do genoma. Para tentar identificar onde é que estas alterações ocorreram, foi realizado um Amplified Fragment Length Polymorphism. Os resultados obtidos permitiram identificar uma sequencia de DNA polimórfica que é altamente repetitiva no genoma de *Vitis vinifera* e encontra-se na vizinhança de um gene (NADH), nos tratamentos HS-0 h e HS-24 h. Foi também demonstrado que o stress térmico influencia o nível de transcrição de várias zonas codificantes e não-codificantes do genoma. PCR quantitativo em tempo real demonstrou que existe um aumento na expressão imediatamente a seguir ao stress térmico, para sequências relacionadas com o *Gret1*. Por outro lado, foi também possível observar uma diminuição da expressão de genes envolvidos na identificação e reparação de quebras duplas no DNA, para o tratamento HS-0 h. Concluindo, os resultados fornecidos por este trabalho, demonstram que o stress térmico afeta tanto a estabilidade genómica como a transcrição.

Palavras chave: *Vitis vinifera*, stress térmico, *Gret1*, alterações genómicas, transcrição.

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1 - Introduction

Global climate change and global warming are currently considered a serious problem and are therefore given a significant amount of attention by the scientific community. It has been estimated that average temperature increased approximately 0.5 °C during the 20th century and that it will rise between 1.4 and 5.8 °C by the end of this century (Wang and Ye, 1995). Temperature stress has thus become an issue for crop plants in the Mediterranean Basin, where most plants are exposed to optimal temperatures at least at some time during their life cycle (Diffenbaugh *et al.*, 2007). The effects of climate change on crop production are expected to significantly increase in the years to come (reviewed in Wahid *et al.*, 2007).

Grape, *Vitis vinifera* L., is an agro-economically important species cultivated worldwide with highly valued products such as fruits, juices, liquors and wines (Vivier and Pretorius, 2002). Similarly to other crop plants, various environmental stresses affect grape development, with temperature being the major determining factor (Cramer *et al.*, 2011). Temperatures at midday can reach above 40 °C in several wine regions, which can threaten berry quality and economic outcomes (Schultz, 2007; Howell, 2001). As temperature increases and water levels decrease, these problems will become more and more relevant (Cramer *et al.*, 2011; Van Leeuwen, 2004; Pereira *et al.*, 2014).

A significant number of studies have investigated the morphological and physiological responses of plants to high temperatures, mainly focusing on photosynthesis, respiration, cell membrane stability, hormone changes and stress oxidation (Liu *et al.*, 2012). The availability of grapevine genome sequences in 2007 (Jaillon *et al.*, 2007; Velasco *et al.*, 2007), permitted for increased approaches involving genomic and functional genomics analysis (Cramer, 2010; Cramer *et al.*, 2011; Liu *et al.*, 2012; Wang *et al.*, 2014). This project aims to identify putative genomic and transcriptomic alterations induced by heat stress in *Vitis vinifera* leaves.

***Vitis vinifera* L.**

V. vinifera is a perennial woody vine belonging to the Vitaceae family whose domestication is believed to have occurred between seven to four thousand years B.C., in the geographical area between the Black Sea and Iran (Châtaignier, 1995; McGovern *et al.*, 1996; Zohary, 2004). From this region, cultivated forms of *Vitis* spread to the Near East, Middle East and Central Europe. As a result, these areas may have constituted secondary domestication centers (Grassi *et al.*, 2003; Arroyo-Garcia *et al.*, 2006). Based on its cultivated area and economic value, grapes are one of the major fruit crops in the world. This is evident by estimates of grape production for wine making worldwide, consisting of 69 093 293 tons of grapes which is equivalent to 39 494 901 US Dollars in 2012 (Food and Agriculture Organization of the United Nations, FAO). Besides wine, which is its major product, grapes are also used for liquor, fresh fruit, dried fruit and juice production. In Portugal, grapes are the most agro-economically relevant crop with a production of 694 612 tons in 2011 (Food and Agriculture Organization of the United Nations, FAO). There is significant genetic variability in Portuguese grapevine, evident as more than 300 genetically distinct cultivars (Almadanim *et al.*, 2007; Gonçalves *et al.*, 2007) used to produce a wide array of different wines (Stevenson, 2005). The traditional Portuguese cultivar Touriga Nacional was chosen as the object of this study. Thought to have originated in the Douro region in the North of Portugal, this traditional cultivar is used throughout the entire country (Instituto da Vinha e do Vinho, I.P.). Despite having a low yield, it's considered to be the queen of Portuguese cultivars due to the high quality wines produced.

Retrotransposons

Mobile genetic elements (MGEs) are ubiquitous genetic units that constitute a significant portion of most plant genomes. There are two major classes of MGEs (Alzohairy *et al.*, 2013), class I and class II elements where transposons are considered Class II elements, and have an RNA independent “cut-and-paste” mechanism (Le *et al.*, 2000). On the other hand, class I mobile elements, or retrotransposons, propagate by a “copy-and-paste” mechanism. Retrotransposition involves complex processes including reverse transcription and integration of a double stranded DNA copy of the mobile element into a new site in the genome (Dombroski *et al.*, 1994; Kumar and Bennetzen, 1999; Wicker and Keller, 2007). Due to their dynamics and mobility, it is widely accepted that this class of mobile DNA elements have a significant role in the evolution of their host genome (Hurst and Schilthuis, 1998; Volf and Brosius, 2007).

There are three types of retrotransposons, as shown in Figure 1.1 (Fávaro *et al.*, 2005). These are; long interspersed elements (LINEs), short interspersed elements (SINEs) and long terminal repeat (LTR) containing elements (Singer, 1982). The latter can be further divided into two superfamilies,

namely Ty3 or *gypsy*-like and Ty1 or *copia*-like, with *gypsy*-like retrotransposons having the closest similarity to retroviruses (Friesen *et al.*, 2001). LTR retroelements contain the *gag* (group-specific antigens) and *pol* (encoding polymerase) genes responsible for structural and enzymatic functions related to retrotransposition flanked by two LTRs. The first gene, *gag*, encodes for products that associate with retrotransposon transcripts to form virus-like particles (VLPs) while the second gene, *pol*, encodes four products essential for proliferation, including *aspartic protease* (*pr/ap-pr*), *reverse transcriptase* (*rt*), *ribonuclease-H* (*rh*) and *integrase* (*in*). *Copia* and *gypsy* type elements are defined by the arrangement of the *reverse transcriptase* and *integrase* domains in their *pol* genes, as illustrated in Figure 1.1 (Fávaro *et al.*, 2005).

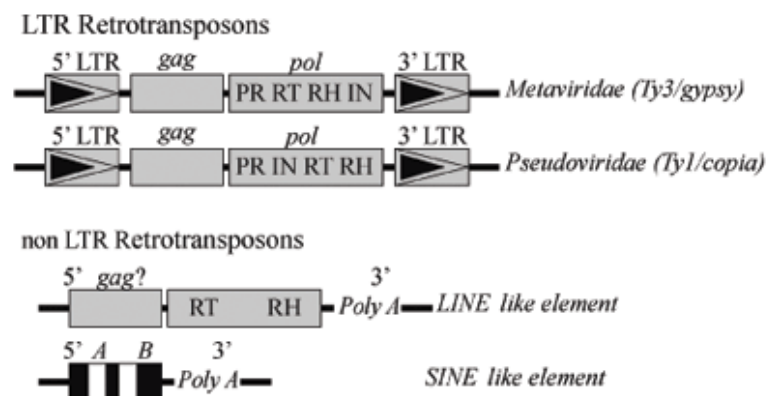


Figure 1.1 – Schematic representation of structural characteristics of retrotransposons. Long terminal repeat (LTR) retrotransposons resemble retrovirus and have LTRs flanking internal domains for *gag* and *pol* retroviral gene products. *Gypsy* type elements differ from *Copia* type elements by the order of the *Reverse Transcriptase* and *Integrase* domains in their *pol* genes. LINE and SINE non LTR-retrotransposons lack terminal repeats and carry a poly (A) tail at their 3' ends. LINEs possess two long open reading frames (ORFs), with similarities to *gag* as well as the *reverse transcriptase* (*rt*) and *ribonuclease-H* (*rh*) genes. SINEs are short elements which contain an internal RNA polymerase III promoter with bipartite structure (boxes A and B). Since SINEs do not encode RT, they rely on this enzyme from other elements for their mobilization. Image altered from Fávaro *et al.*, 2005.

Once considered to be “junk DNA” mobile elements are now known to be an important component of genomes, affecting various processes from chromatin structure and organization to gene expression (reviewed in, Hodgetts, 2004). In plants, contrary to animals, the most frequent types of mobile DNA elements are *gypsy* and *copia* like LTR containing retrotransposons. Despite the difference in the *pol* gene organization of *copia*-like and *gypsy*-like LTR retrotransposons their mechanism of propagation is the same, and is illustrated in Figure 1.2 (Havecker *et al.*, 2004). Due to their activity and mode of propagation, these types of elements have accumulated in plants genomes, representing approximately 20 % of the rice genome, and up to 90 % of large genomes such as that of wheat (Flavell, 1986; Jianxin *et al.*, 2001). The molecular characterization and cytological localization of retrotransposons containing long terminal repeats (LTR) have been studied in numerous plant species, and their distribution varies depending on the element type as well as the genome in

question. In species with large genomes, *gypsy*-like and *copla*-like LTR retroelements are disproportionately abundant in heterochromatin, including pericentromeric and terminal heterochromatic regions (Belyayev *et al.*, 2001; Wong and Choo, 2004). However, there is also evidence of retroelements dispersed or clustered throughout gene rich regions (SanMiguel *et al.*, 1996; Sandhu and Kulvinder, 2002).

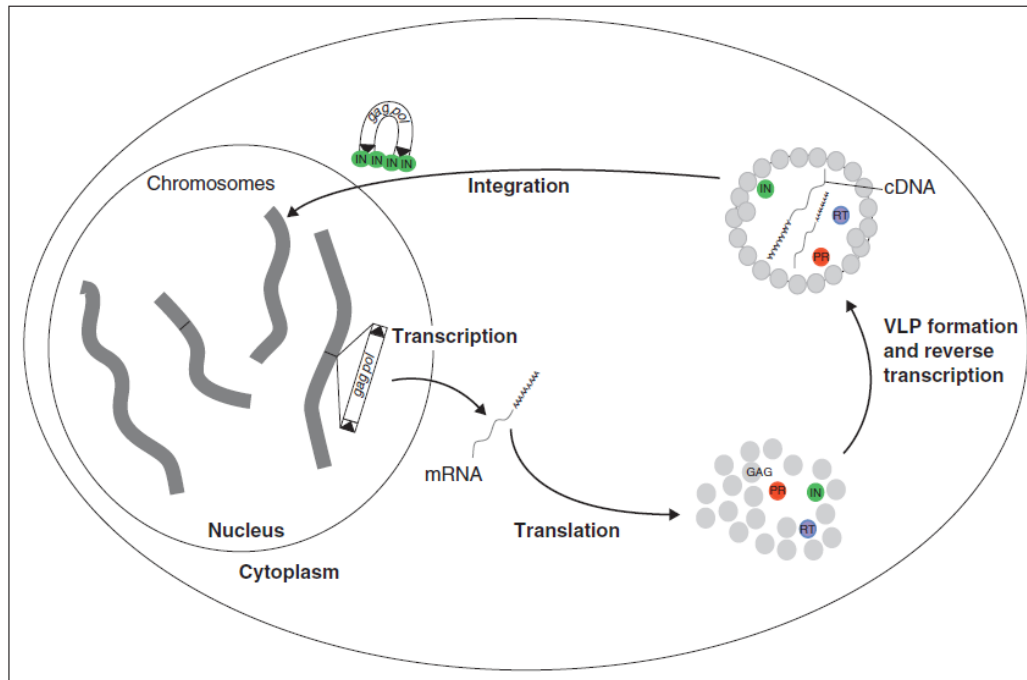


Figure 1.2 – The life cycle of LTR retrotransposons. LTR retrotransposons contain LTRs at both ends of the *gag-pol* coding region as shown by black triangles and propagate by a “copy and paste” mechanism. The *gag-pol* coding region encodes gene products which are active in the cytoplasm as shown by circles, including INTEGRASE (IN-green), ASPARTIC PROTEASE (PR-red), REVERSE TRANSCRIPTASE (RT-blue), and glycosaminoglycan (GAG-grey). In the cytoplasm, GAG proteins form virus-like particles (VLP) where retrotransposon specific mRNAs and proteins are captured and reverse transcription of the viral mRNAs occurs to generate DNA intermediates. These DNA intermediates remain associated with INTEGRASES as they exit the VLP, are imported into the nucleus, and integrated into the genome. Image adapted from Havecker *et al.*, 2004.

Due to their behavior and their genomic distribution, retrotransposons can be utilized as powerful molecular tools of genetic diversity and evolution. The *gag* and *pol* regions are highly conserved, thus allowing for easy detection and analysis at the genetic as well as cytogenetic level. LTR retrotransposons can be further explored via their LTR sequences, which are typically between 0.5 and 2 kb and are highly conserved within specific retrotransposons (Llorens *et al.*, 2011). Considering that the exact same sequence between the 5`-LTR and 3`-LTR is an essential characteristic for genomic insertion, sequence divergence between LTRs is often used for evolutionary studies (Llorens *et al.*, 2009). Other PCR based techniques that take advantage of LTR sequences are retro-element-microsatellite-polymorphism-amplified (REMAP) and inter-retroelement-amplified-polymorphism (IRAP) (Kalendar *et al.*, 1999; Kumar and Hirochika, 2001; Schulman *et al.*, 2004).

These two procedures have been used as molecular tools for a variety of studies in plants, such as genetic mapping (Manninen *et al.*, 2000), study of genetic stability in aploidiploid species (Baumel *et al.*, 2002), creation of phylogenetic trees (Breto *et al.*, 2001), study of genetic diversity among crops (Pereira *et al.*, 2005), among others.

Grapevine Retrotransposon 1 (*Gret1*)

Similarly to other plant species, a significant portion of the *Vitis vinifera* genome can be attributable to mobile DNA elements such as retrotransposons. An early estimate based on a BAC library constructed from the cultivar Syrah showed 41 % to be retroelement related (Tomkins *et al.*, 2001). This is in accordance with more recent calculations obtained with whole genome sequencing efforts, where transposable DNA elements are more abundant in grapevine than in *Arabidopsis*, poplar or rice (Jaillon *et al.*, 2007; Velasco *et al.*, 2007). In one publication, 108.5 of the estimated 504.6 Mb haploid nuclear genome is transposable element related, including class I (retrotransposons: *copia*, *gypsy*, LINE) and class II (DNA transposons: *Mutator*, CACTA, hAT) (Velasco *et al.*, 2007). The most abundant MGEs were *gypsy/athila*-like elements followed by *copia*-like elements. In a separate grapevine genome sequence, 41.4 % of the grapevine genome was estimated to be composed of repetitive/transposable elements (Jaillon *et al.*, 2007). Both publications indicate a large prevalence of class I mobile elements, or retrotransposons, which are distributed unevenly along the chromosomes along with other repetitive sequences. To date, a number of retrotransposons have been characterized in *V. vinifera*, including *Gret1*, *Vine-1* and a number of *Tvv1/copia*-like elements (Kobayashi *et al.*, 2004; Verriès *et al.*, 2000; Moisy *et al.*, 2008, respectively).

In this work, *Gret1* related sequences were utilized to investigate the effects of heat stress on genomic integrity and function. This LTR retrotransposon was initially discovered as an insertion mutation conferring white berry phenotype in grape (Figure 1.3). The skin color of the berry is determined by the quantity and composition of anthocyanins, so that black (including red and purple) cultivars accumulate anthocyanins in their skins, while white (including yellow and green) cultivars do not. In *Vitis*, anthocyanins biosynthesis is controlled by a gene for *UDP-glucose: flavonoid 3-O-glucosyltransferase* (Kobayashi *et al.*, 2002), namely *VvmybA1* in *V. vinifera* (Kobayashi *et al.*, 2004; Kobayashi *et al.*, 2005). *Gret1* was initially characterized as an insertion mutation of the *VvmybA1* gene. These mutations, evident in the *VvmybA1a* and *VvmybA1b* alleles, contain a complete or partial *Gret1* retrotransposon upstream of the *VvmybA1*-coding sequence, respectively. This, in turn impedes *VvmybA1* transcription leading to white grape phenotype when homozygous.

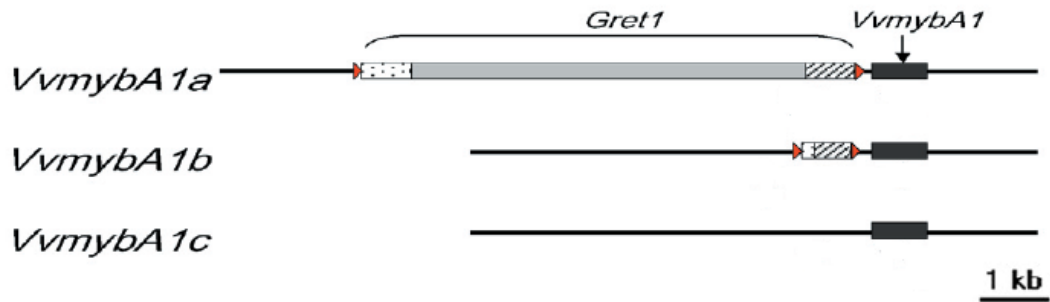


Figure 1.3 – *Gret1* was initially identified as a mutation of the *VvmybA* gene affecting grape skin color. *VvmybA1a*, *VvmybA1b*, and *VvmybA1c* alleles are shown where the *VvmybA1a* allele has a complete *Gret1* sequence inserted upstream and *VvmybA1b* possesses a portion of the *Gret1* LTR (box with diagonal strips). Both *VvmybA1a* and *VvmybA1b* result in no *VvmybA* product, resulting in plants which lack of color in grape skin (white grapes) if homozygous. Plants with one copy of the third allele, *VvmybA1c* have colored (red or purple) grapes. Figure altered from Kobayashi *et al.*, 2007.

Based on its 10 422 base pairs (bp) long sequence, *Gret1* can be classified as a *gypsy*-type retrotransposon with two 824 bp LTRs flanking an internal 8 774 bp region (Kobayashi *et al.*, 2004). Since its discovery, the popularity of *Gret1* is evident by the dozens of entries of associated sequences in the core nucleotide records, not only for *V. vinifera* but also for other species such as *V. riparia*, *V. aestivalis*, and *V. lambrusca*. Previous work done in our laboratory showed that the *reverse transcriptase* domain (*rt*) of *Gret1* is highly conserved and that this element localizes to euchromatic or gene rich regions of the genome, suggesting a possible role in gene expression (Pereira *et al.*, 2005). Furthermore, REMAP and IRAP molecular marker techniques with primers based on *Gret1* LTR sequences indicated that there are differences in the genomic distribution of these retrotransposons between cultivars but not within clones of the same cultivar (Pereira *et al.*, 2005). Taken together, these results indicate that *Gret1* can be used as a molecular tool to identify cultivars and that the genomic distribution of this retrotransposon may have a role in the phenotypes that define a cultivar.

Heat stress

As sessile organisms, plants have evolved complex adaptation and/or acclimation mechanisms to deal with environmental stresses, including high temperatures. Depending on exposure time and actual temperature, heat stress can be chronic, as experienced in hotter habitats, or acute, as a consequence of seasonal or daily extreme temperatures. High temperature effects also depend on plant tissue and developmental stage. Therefore, heat stress and its respective tolerance are not a single occurrence, but rather a varying set of complex perturbations to the organism's homeostasis (Larkindale *et al.*, 2005).

Effects of heat stress at the cell level

Heat affects a variety of structures, functions and processes at the cellular level. Considering that proteins work at an optimal temperature range, temperature increases can alter enzyme activity and lead to deregulation of metabolic pathways, and in extreme conditions to their denaturation. Besides effects on the tertiary structure of individual proteins, heat stress also incurs considerable defects on cytoskeleton structure and function. It has been shown that a slight increase in temperature can induce reorganization of actin filaments into stress fibers (Toivola *et al.*, 2010), whereas severe heat stress results in aggregation of filament-forming proteins and the collapse of actin and tubulin networks (Welch and Suhan, 1985; Welch and Suhan, 1986). Golgi apparatus and endoplasmic reticulum (ER) function is also impaired by heat, evident as a decline in the number of lysosomes and mitochondria (Welch and Suhan, 1985). Furthermore, decreased oxidative phosphorylation combined with the loss of mitochondria creates a drop in ATP levels during heat stress (Lambowitz *et al.*, 1983; Patriarca and Maresca, 1990).

Heat stress also affects various nuclear and cytoplasmatic processes. The majority of RNA splicing is strongly affected (Vogel *et al.*, 1995) and translation machinery is compromised, resulting in incorrectly processed ribosomal RNAs and aggregates of ribosomal proteins (Welch and Suhan, 1985; see related review in this issue by Boulon *et al.*, 2010). Heat stress has also been found to induce the formation of stress granules, which are large RNA-protein structures containing mRNAs, translation initiation components, and other proteins related to mRNA function in the cytosol (Nover *et al.*, 1989; Buchan and Parker, 2009). These granules are believed to be caused by the inhibition of protein synthesis, one of the earliest metabolic responses to heat (Good and Zaaplachinski, 1994). In addition to proteins and RNAs, high temperatures alter lipid properties. This results in cell membranes becoming more fluid and permeable, which in turn changes ion homeostasis and decreases cytosolic pH (Coote *et al.*, 1991; Piper *et al.*, 2003). Taken together, these heat induced effects can lead to cell cycle arrest and growth stagnation (Lindquist, 1980; Yost and Lindquist, 1986; Zeuthen, 1971). Depending on the length and severity of the heat stress, the accumulation of heat effects can result in cell death.

Although organisms have different optimal temperature ranges, they all have a highly conserved defense mechanism to high temperature, known as the heat stress response. At the molecular level, the most studied and known characteristic of the heat stress response is the induction of the expression of a set of highly conserved proteins, known as heat stress proteins (HSPs) (Welch *et al.*, 1991; Richter *et al.*, 2010). The majority of these, such as HSP70 and HSP90, function as molecular chaperones facilitating protein folding and assembly (Gething and Sambrook, 1992; Hartl, 1996). On the other hand, other HSPs such as HSP100 belong to the AAA+ family of ATPases that are involved in resolubilizing protein aggregates (Bösl *et al.*, 2006). All HSP transcription is regulated by

Heat Stress Factors (HSFs), which are constitutively expressed in higher eukaryotes and are activated under high temperature conditions (Sarge *et al.*, 1993; Anckar and Sistonen, 2011).

Through transcriptional profiling or proteomic analyses, the heat stress response has been addressed on a genome-wide level in a variety of cells and organisms (Eisen *et al.*, 1998; Gasch *et al.*, 2000, GuhaThakurta *et al.*, 2002; Matsuura *et al.*, 2010; Rohlin *et al.*, 2005; Tabuchi *et al.*, 2008; Liu *et al.*, 2012). These studies showed a conserved upregulation of 50 to 200 genes correlated to heat stress response in a variety of model organisms, such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Archaeoglobus fulgidus* and *Arabidopsis thaliana* (Gasch *et al.*, 2000; Tabuchi *et al.*, 2008). In grapes, approximately 8 % of the total leaf transcriptome was found to be responsive to heat stress and subsequent recovery (Liu *et al.*, 2012). In this study, there was heat induced up regulation of a large number of genes involved in essential biological pathways, including cell rescue (i.e., antioxidant enzymes), protein fate (i.e., HSPs), primary and secondary metabolism, transcription factors (i.e., HSFs), signal transduction and development. Interestingly, the proportion of heat stress-regulated genes immediately upon stress exposure was almost twice than those involved in recovery, likely due to diverse expression kinetics of individual heat-induced genes (Liu *et al.*, 2012). This is in accordance with previous studies showing that the expression of fast-responding genes involved in rapidly counteracting heat induced damages can vary from those involved in recovery (Eisen *et al.*, 1998). For example, chaperone genes encoding for heat shock proteins are among the most substantially upregulated, becoming a major constituent of total cytosolic protein levels during heat stress (Phipps *et al.*, 1991). On the other hand, genes important for cell organization, DNA/ RNA repair, and some metabolic processes, tend to be expressed during the adaptation or recovery phases (Eisen *et al.*, 1998; Gasch *et al.*, 2000).

Heat stress effects on retrotransposon related sequences

Due to their latent mutagenic capacity, mobile DNA elements can represent a threat to the integrity and stability of their host genomes (Kidwell and Lisch, 2000). Plants have therefore evolved complex mechanisms of transposable element repression during their evolution. Due to their “copy and paste” mechanism of propagation, retrotransposon transcription is the first step towards retrotransposition and is therefore highly regulated. Accordingly, a number of regulatory networks of epigenetic silencing effective against transcriptional activity of numerous different MGEs are known. For example, silent elements are typically associated with high levels of DNA methylation at cytosines, and histone modifications including methylation at lysine 9 of histone H3 (H3K9me2) (Lisch, 2009; Slotkin and Martienssen, 2007). A reinforcing loop of mobile element silencing machinery also exists where RNA directed DNA methylation (RdDM) is lead by the expression of 24 nt small interfering RNAs (siRNAs) (reviewed in Zhang and Zhu, 2011).

Various abiotic stresses such as heat, chilling and mechanical damage have been shown to impair LTR Retrotransposons transcriptional silencing (Cavrak *et al.*, 2014; Hirochika, 1995; Grandbastien *et al.*, 2005; Salazar *et al.*, 2007). For example, a recent study demonstrated heat induced transient loss of transcriptional gene silencing in retrotransposon associated sequences in *Arabidopsis* (Pecinka *et al.*, 2010). This reduction appeared to be independent of methylation levels but rather associated with a genome wide loss of nucleosome density. More evidence of the role of retrotransposons in stress response comes from several endogenous stress promoters sharing strong sequence similarities with LTRs (White *et al.*, 1994). In fact, some plant elements have *cis*-regulatory elements associated with plant defense response mechanism in their promoter (U3 region of LTR 5') (Salazar *et al.*, 2007; Cavrak *et al.*, 2014). Since retrotransposon promoter elements are targeted by similar defense-induced transcription factors as those of plant defense genes, their transcriptional activity can be activated by stress conditions (Casacuberta and Santiago, 2003). This is evident in heat stress transcription activation of a Ty1/*cop*ia-type LTR Retrotransposon named *ONSEN* (ATCOPIA78) in *Arabidopsis* (Pecinka *et al.*, 2010; Tittel-Elmer *et al.*, 2010). Transcription of *ONSEN*-related sequences after heat stress was also observed in most species of the Brassicaceae (Ito *et al.*, 2013), demonstrating a conserved mechanism of activation

Besides heat induced transcriptional activation of retrotransposons, a recent publication described heat induced retrotransposition of *ONSEN* (Cavrak *et al.*, 2014). Not only was there heat activation transcription of this retrotransposon by a heat-responsive element present within its own sequence, full length extrachromosomal DNA was found under elevated temperatures. Interestingly, extrachromosomal *ONSEN* DNA was found to be capable of reintegrating into the genome, which was especially obvious in dividing cells from the meristematic tissue of the shoot. Therefore, although retrotransposons are usually transpositionally inactive, there are few that have held their capacity to transpose and are activated only under stress situations (Kumar and Bennetzen, 1999; Cavrak *et al.*, 2014).

DNA damage and double-strand breaks

The genome of plants is under constant attack from endogenous and exogenous DNA damaging factors, such as reactive radicals, radiation, and genotoxins (reviewed in, Yoshiyama *et al.*, 2013). As described above, heat stress can cause numerous deleterious effects on the cell, thus being a relevant source of possible DNA damage. Heat induced DNA lesions can be in the form of single strand breaks (SSBs) and/or double strand breaks (DSBs). During the past 10 years, several research groups have shown that heat stress can induce DNA DSBs (Kaneko *et al.*, 2005; Hunt *et al.*, 2007; Takahashi *et al.*, 2008; Laszlo and Fleischer, 2009b). In comparison to SSBs, DSBs are considerably

more damaging as the cell has no intact strand to direct repair. DSBs can therefore lead to significant karyotypic instability and possible cell death (Bennett *et al.*, 1993; Bennett *et al.*, 1996).

For damage control and subsequent repair, DSBs detection by distinct sensor proteins is essential, with MRE11/RAD50/NBS1 (MRN) complex being the initial sensor of DSBs in both plants and animals. Once DSBs are detected by this complex, the ATAXIA TELANGIECTASIA MUTATED (ATM) pathway is triggered at the DSB sites (Rupnik *et al.*, 2010). Subsequently, phosphorylation of specific targets by ATM kinase activity occurs, including Ser-139 in the C-terminus of H2AX, which is a variant of the core histone H2A (Dickey *et al.*, 2009). Immunocytochemically, it was shown that a single γ H2AX focus corresponded to one DSB (Sedelnikova *et al.*, 2002). Relevantly, heat has been shown to stimulate the phosphorylation of histone H2AX, reinforcing the idea that the increase of heat causes DSBs (Kaneko *et al.*, 2005; Hunt *et al.*, 2007; Takahashi *et al.*, 2008; Laszlo and Fleischer, 2009a,b).

DNA double strand-break (DSB) repair mechanism

Once DNA DSBs are detected by the cell, a number of complex processes occur to recruit the machinery necessary for subsequent damage repair. There are three well conserved repair mechanisms for DNA double strand breaks in eukaryotes, as shown in Figure 1.4 (Kim *et al.*, 2013). The first mechanism of repair, non-homologous end joining (NHEJ), is active during G₀/G₁ and early S phases and involves direct ligation of DNA end breaks. Since this pathway is independent of a homologous template, it is highly prone to error (Bleuyard *et al.*, 2005). The second repair mechanism, namely microhomology mediated end joining (MMEJ), shares repair proteins with the NHEJ pathway but utilizes a short (5-25 base pairs) homologous sequence to align the broken strands before joining (Bleuyard *et al.*, 2005). Contrary to NHEJ, MMEJ repair is known to occur mostly during the S-phase of the cell cycle (reviewed in Yoshiyama *et al.*, 2013). Homology-directed repair, or homologous recombination (HR), is the third mechanism for DSBs damage repair (Figure 1.4). HR occurs mainly during late S and G₂ phases of the cell cycle after replication of the genome since it depends on homologous chromosome alignment (Kimura and Sakaguchi, 2006). This mechanism requires an extensive 5'-3' portion of DNA to generate a 3' single-stranded tail, which is then displaced by the RAD51 recombinase and forms a RAD51/ssDNA nucleofilament that invades the homologous DNA double strand molecule (Dudas and Chovanec, 2004; Symington, 2002). This process, named strand exchange, forms a DNA crossover or Holliday junction which provides a primer to initiate new DNA strand synthesis (Dudas and Chovanec, 2004; Symington, 2002; Krogh and Symington, 2004).

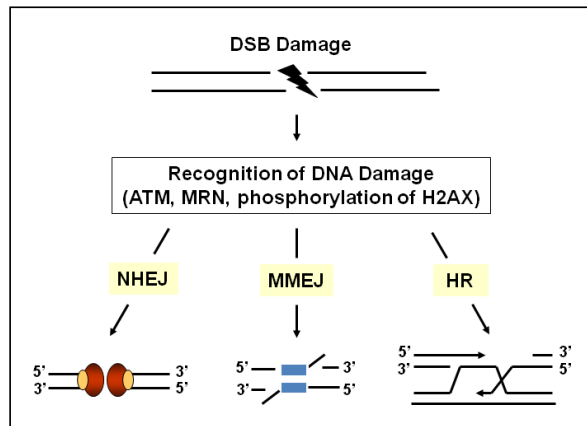


Figure 1.4 – Three main DNA double-strand break (DSB) repair mechanisms in eukaryotes. DSBs are recognized by MRN complex and ATM, leading to phosphorylation of H2AX. Depending on cell cycle, DSB repair can occur through nonhomologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), or homologous recombination (HR) repair pathways. Figure adapted from Kim *et al.*, 2013.

In the majority of mitotically inactive cells, the DNA DSBs are repaired by a non-homologous end joining (NHEJ) pathway (reviewed in Yoshiyama *et al.*, 2013). This mechanism requires a coordinated assembly of damage-responsive proteins at the damage site for direct rejoining of the separated DNA breakpoints, as shown in Figure 1.5. In NHEJ, DSB repair is initiated by binding of the Ku70-Ku80 complex to the DSB ends and recruitment of DNA-dependent protein kinase catalytic subunit (DNA-PKcs), a 465-kDa ser/thr kinase that mediates synapsis of DNA breakpoints prior to auto-phosphorylation (Hartley *et al.* 1995). This is followed by the recruitment of Artemis, a nuclease and kinase/phosphatase (Caldecott, 2008; Pannicke *et al.*, 2004; Ma *et al.*, 2005). Lastly, the recruitment of the X-RAY REPAIR CROSS-COMPLEMENTING PROTEIN 4 (XRCC4) in conjunction with DNA Ligase IV (Lig4) forms a complex, which is essential for the final step of DNA breakpoint end joining (Grawunder *et al.*, 1997; Critchlow *et al.*, 1997).

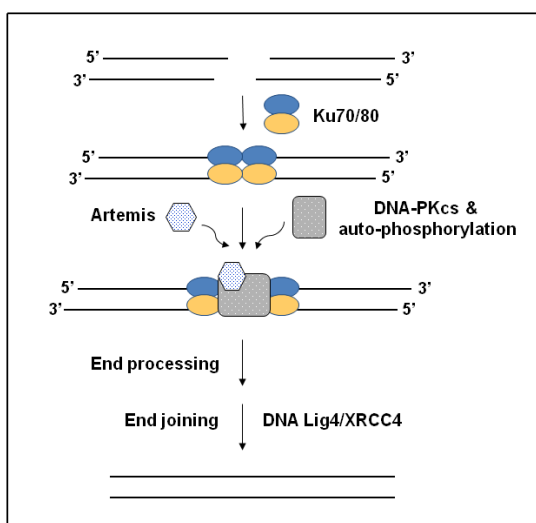


Figure 1.5 – Non-homologous end joining (NHEJ) DNA repair pathway. DSB repair is initiated by binding of the Ku70-Ku80 complex to the DSB ends, resulting in conformational changes that allow binding of DNA-PKcs, a DNA-dependent protein kinase catalytic subunit that mediates synapsis of DNA breakpoints prior to auto-phosphorylation. This is followed by the recruitment of Artemis, and subsequently by the XRCC4-DNA ligase IV (Lig4) complex for the final step of DNA end joining. Figure adapted from Kim *et al.*, 2013.

2 - Materials and Methods

2.1 - Plant Material and Heat Treatments

Vitis vinifera L. Cv. Touriga Nacional canes were collected from an experimental population in Northern Portugal (Quinta da Leda, Douro), and maintained for approximately 3 months at 4 °C in complete darkness until use. Heat stress treatment was performed as illustrated in Figure 2.1.1 and described below.

2.1.1 - Controlled growing conditions

In order to obtain leaves for posterior molecular analysis, dormant canes were removed from the cold room at 4 °C and the proximal portions cut into approximately 20 cm shoots. These were cautiously washed in 3 % (v/v) bleach, placed in water and allowed to develop roots, leaves and inflorescences in a growth chamber with a 16 h light (24 °C)/8 h dark (20 °C) cycle for approximately 2 to 3 weeks, until the majority of leaves contained diameters of 1-2 cm. For collection of control leaf material, young leaves (< 2 cm diameter) were removed from untreated canes simultaneously with corresponding heat stress samples and designated as Control 0 hours (C-0 h) and Control 24 hours (C-24 h).

2.1.2 - Heat stress conditions

The heat stress treatment was performed as previously described in Pereira *et al.*, 2014 and shown in Figure 2.1.1. Plants with growing roots, leaves and inflorescences were transferred from controlled growing conditions and placed at 42 °C for 4 h in controlled humidity. After heat stress, leaves with 1 to 2 cm diameter were either collected immediately (HS-0 h) or canes were returned to controlled growing conditions for further 24 h before collection (HS-24 h).

Young leaves (<2 cm diameter) from both control and heat stressed plants were collected, immediately frozen in liquid nitrogen and stored at -80 °C for subsequent extraction of genomic DNA or RNA for molecular analysis. Once leaves were removed from developing plants, the respective cane was discarded and not used for further analysis.

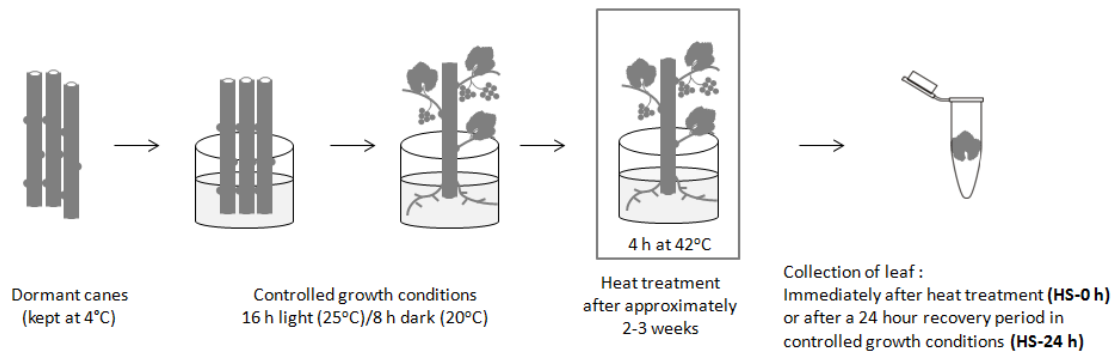


Figure 2.1.2 – Heat stress treatment. Dormant canes maintained at 4 °C were transferred to controlled growth conditions (16 h light (25 °C)/8 h dark (20 °C)) for approximately 2-3 weeks. For heat stress treatment, plants were exposed to 42 °C for 4 hours at controlled humidity. Young leaves were collected right after the heat treatment (HS-0 h) or returned to controlled growing conditions for 24 h before collection (HS-24 h). Figure adapted from Pereira *et al.* 2014.

2.2 - DNA Extraction

For genomic analysis, DNA was extracted utilizing the CTAB method (Thomas *et al.*, 1993), as routinely performed in the laboratory and described in detail in (S 1). Approximately 1 g of frozen leaf tissue from the control or stressed plants was ground to a fine powder in liquid nitrogen for DNA extraction. After extraction, genomic DNA integrity was analyzed by 1 % (w/v) Agarose gel electrophoresis and its concentration and purity determined via Nanodrop.

2.3 - Analysis of *Gret1* copy number by Real-Time PCR

Quantitative Real Time PCR (qRT-PCR) with the Bio-RAD IQ5 Multicolor Real-Time PCR Detection System was utilized to quantify the number of copies of *Gret1* related sequences in control plants or plants that had undergone heat stress treatment. The set of primers used is shown in Table 2.3. The SSR locus *VVS2* is present as a single copy in the *V. vinifera* haploid genome and was therefore used as a reference for titration curves (Jaillon *et al.*, 2007; Velasco *et al.*, 2007). Touriga Nacional has been shown to have a nuclear genome content of 0.61 pg per haploid genome (Leal *et al.*, 2006). This estimate was utilized to perform serial genomic DNA dilutions containing 100 000 genome copies (61 ng), 10 000, 5 000, 1 000, 500 and 100 copies of the genome per µl (each dilution

was done in triplicate). To quantify the number of *Gret1* related sequences, three sets of primers specific for different segments of the *Gret1* LTR (LTR1, LTR2 and LTR3) and one primer pair specific for the coding sequence of the *reverse transcriptase* (*rt*) domain of the *pol* gene were utilized.

The BIO-RAD kit IQ SYBR Green Supermix (Bio-Rad Cat # 170-8880S, Hercules, CA, USA) was utilized for PCR on genomic DNA from control plants and stressed plants as described in the manufacturer's protocol. Each 20 µl reaction mixture contained: 1 µL of DNA, 10 µL of SYBR and 20 pmol of each primer. The program of qRT-PCR and their respective conditions were followed as described in (Pereira *et al.*, 2009). Cycle conditions used were: 25 cycles (95 °C 5 min, 35 cycles of 95 °C, 1 min; 55 °C, 1 min; and 72 °C, 1 min, and a final elongation step of 72 °C for 5 min).

For analysis of number of copies, only reactions with dissociation curves with unique peaks and expected dissociation temperatures were used. Titration curves were calculated for control and stressed plants using the amplicon of the *VVS2* as a reference and triplicates for each DNA dilution. For this, average *Ct*-values of each dilution on the x axis and logarithm number of copies on the y axis were plotted thereby allowing for estimation of a regression line ($\log \# \text{ copies} = x (\text{the average } Ct\text{-value}) + a$, where R^2 was fixed as always higher than 0.9. Replacing the *b* in the regression line with the *Ct*-value mean of each sequence, will give the number of copies present in the genome of each segment amplified (Table 2.3).

Student's t-test (two-tailed distribution , type 3- unequal variance) was used to analyze putative effects of heat stress on the number of *Gret1* related sequences between control and heat treated plants (HS-0 h and HS-24 h) . All data was analyzed separately for biological and experimental replicates with the program Microsoft® Office Excel 2007.

Table 2.3 – Primers utilized in the qRT-PCR to quantify the *Gret1* related sequences.

Primers	Sequence
<i>Gret1</i> LTR1-forward	5`-GACACGCAGCACGTGTTATC
<i>Gret1</i> LTR1-reverse	5`-ATGAAGGTTGTCCGGATGTC
<i>Gret1</i> LTR2-forward	5`-GGGTCGAGGTGACAACAAGT
<i>Gret1</i> LTR2-reverse	5`-GAGGATCCCCTTCTTCTTCG
<i>Gret1</i> LTR3-forward	5`-CATGGCTAACAAAACCATCG
<i>Gret1</i> LTR3-reverse	5`-TGTTACCTCGCGTCTTTGG
<i>Gret1</i> -RT-forward	5`-CGAGTTTGTGTAGATTACAC
<i>Gret1</i> -RT-reverse	5`-GCATTTAGAAGGATTTAGCTT

2.4 - REMAP and IRAP

REMAP and IRAP PCR based techniques were realized as previously described in Pereira *et al.* 2005 with primers shown in Table 2.4, which were designed to amplify genomic regions up or downstream of *Gret1* LTRs and/or flanking simple sequence repeats (SSR) as shown in Figure 2.4. In IRAP, PCR products are amplified from two nearby retrotransposons (*Gret1* LTRs) using outward-facing primers. In REMAP, amplification products include genomic regions between retrotransposons as well as those proximal to simple sequence repeats (SSRs).

IRAP and REMAP PCRs were performed in 20 μ L reaction mixtures containing 50 ng of genomic DNA with the following cycling conditions, 25 cycles (95 $^{\circ}$ C 5 min, 25 cycles of 95 $^{\circ}$ C, 1 min; 65 $^{\circ}$ C, 1 min; and 72 $^{\circ}$ C, 1 min, and a final elongation step of 72 $^{\circ}$ C for 5 min). PCR products were separated by 1 % (w/v) agarose gel electrophoresis.

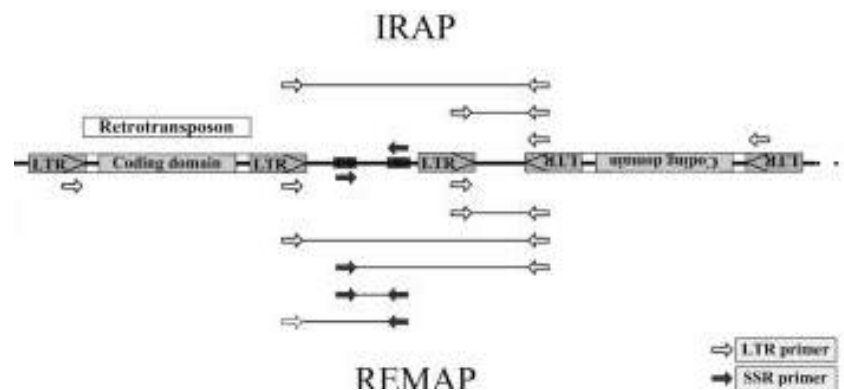


Figure 2.4 – IRAP and REMAP PCR. In IRAP, LTR primers (white arrows) amplify genomic DNA flanking retrotransposons or loose LTRs in opposite orientations. In REMAP, LTR primers are utilized with SSR primers (black arrows), resulting in amplification of genomic regions flanking retrotransposons or loose LTRs in opposite orientations and SSRs. Altered from Bento *et al.*, 2008

Table 2.4 - Primers utilized for *Gret1* based REMAP and IRAP.

REMAP e IRAP	Sequence
<i>Gret1</i> LTR-forward	5`-CAACTAATTTATATTCCTCTCTAACCA
<i>Gret1</i> LTR-reverse	5`-CGTGTTTCCCAGAGGGAGGGGTCCCTAC
Microsat-GA	5`-(GA) ₉ C
Microsat-CT	5`-(CT) ₉ G

2.5 - Southern Blotting

Southern Blotting was performed to analyze putative effects of heat on the organization of *Gret1* related sequences in the genome. For this, genomic DNA (5 µg) of each sample was digested with isoschizomer restriction enzymes *HpaII* or *MspI*, according to manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Both enzymes cut the sequence CCGG, however unlike *HpaII*, *MspI* can cleave the sequence when the internal C residue is methylated. Digested DNA was then separated by 1 % (w/v) agarose gel electrophoresis and subsequently transferred to a membrane Hybond N + (Amersham, Piscataway, NJ, USA) using the alkaline method. For this, Agarose gel containing genomic DNA was placed in 0.25N HCl to denature the DNA prior to capillary transfer in an alkaline environment to improve the binding to the membrane. Membranes containing DNA were exposed to UV radiation for 5 min in order cross-link DNA to the membrane. Probe hybridization and detection was performed with non-radioactive chemiluminescent ECL, following manufacturer's instructions (Amersham, Piscataway, NJ, USA), with slight modifications in the concentration of the LTR2 probe utilized. The LTR2 has 237 base pairs and has no recognition sites for the *HpaII* or *MspI* enzymes according to the complete *Gret1* published sequence (Kobayashi et al., 2004). Instead of the suggested concentration, we utilized 5ng LTR2 specific DNA per cm² of membrane surface area. Probe for *HindIII* digested λ DNA was utilized as described for molecular weight marker and technical control. All other methodologies, including hybridization with 82 % stringency (0.3 M NaCl), washing and subsequent detection were carried out following manufacturer's instructions.

2.6 - Whole Genome analysis using Amplified Fragment Length Polymorphism (AFLP)

2.6.1 - AFLP Technique

AFLP was performed as described in the manufacturer's instructions with a few modifications (AFLP Analysis System II, Cat. # 10717-015, Invitrogen-Life Technologies, Carlsbad, CA, USA). As illustrated in Figure 2.6.1, the first step was the digestion of genomic DNA with two restriction endonucleases, *EcoRI* and *MseI*, which have a 6 bp and a 4 bp recognition site respectively. Together these two enzymes generate small DNA fragments (< 1000 bp) which can be utilized for subsequent amplification. After heat inactivation of the restriction endonucleases, fragmented DNA was incubated with *EcoRI/MseI* adapters and T4 DNA ligase. The result is adaptor sequences flanking the genomic DNA sequences, which can be utilized as primer binding sites for the next amplifications

Next, pre-amplification PCR was performed with the fragmented genomic DNA containing the ligated adapters and an *MseI* primer (with one selective nucleotide) and an *EcoRI* primer (with no selective nucleotide). The PCR reaction was carried out in 51 µL reactions containing 250 ng of DNA

using the following cycling parameters: 30 cycles of 30 sec at 94 °C and 60 sec at 56 °C and 60 sec at 72 °C. Due to primer design and amplification techniques, the *EcoRI-MseI* fragments are preferentially amplified, rather than *EcoRI-EcoRI* or *MseI-MseI* containing fragments. The pre-amplification product was diluted and stored for selective amplifications.

The second PCR amplification, called selective amplification, primers with selective nucleotides in the 5' extremity (three for the *MseI* and two for the *EcoRI*) are utilized to significantly reduce the number of fragments amplified (between 10 to 100). In this work, primers Mse-CTT, Mse-CAA, Mse-CAC, Mse-CAT, Mse-CTG Eco-TC, Eco-TG, Eco-AG, Eco-TA, Eco-TT were used for selective amplification. Reaction conditions were 12 cycles with annealing temperatures starting at 65 °C and decreasing by 0.7 °C in each cycle until 56 °C followed by a further 23 cycles with 56 °C annealing temperature.

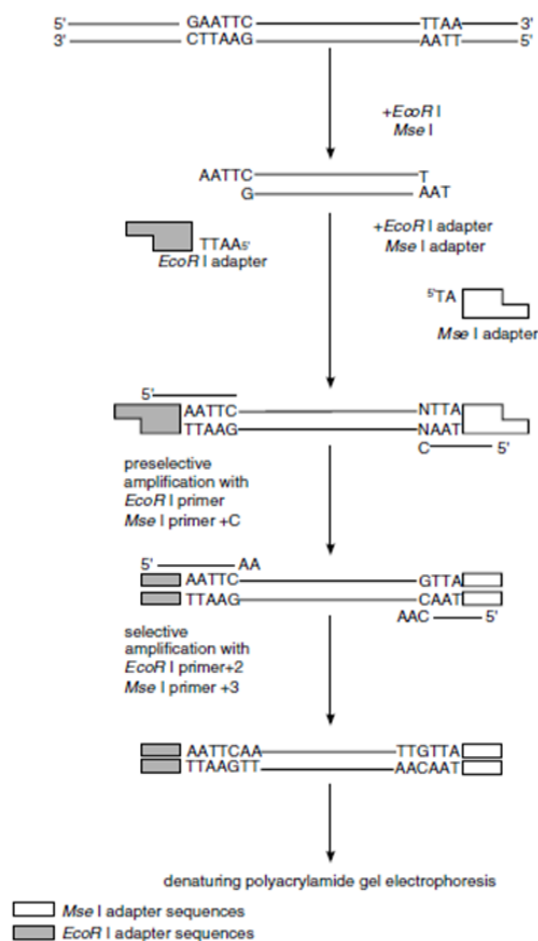


Figure 2.6.1 – Amplified Fragment Length Polymorphism (AFLP) procedure. DNA is fragmented with the restriction endonucleases *EcoRI* and *MseI* followed by ligation of correspondent adapters to the ends of each fragment. Pre-amplification PCR selectively amplifies fragments with the adapters flanking genomic DNA with one selective nucleotide in the primer for the *MseI* adapter. For selective amplification, PCRs are performed in order to reduce the number of amplified fragments with primers containing 2 and 3 selective nucleotides for the *EcoRI* or *MseI* primers respectively. Figure altered from AFLP Analysis System II, Invitrogen.

2.6.2 - Separation of AFLP fragments by Denaturing Polyacrylamide gel electrophoresis

AFLP fragments were analyzed by denaturing polyacrylamide electrophoresis with a Sequi-Gen Nucleic Acid Electrophoresis Cell (Bio-Rad Cat #165-3863, Hercules, CA, USA) which supports a 38 x 50 cm (width x length) glass plate. For each run, glass plates were carefully cleaned and treated with 1.5 mL of the bind silane solution (1.5 μ L bind silane + 1425 μ L ethanol + 75 μ L acetic acid) and 2 mL of repel silane in the glass plate from the electrophoresis structure. For gel setting, the apparatus was assembled with 0.4 mm spacers and a 6 % (v/v) polyacrylamide gel with 7.5 M urea, and 1x TBE buffer (0.89 M Tris, 0.89 M Borate, 0.020 M EDTA) was injected and allowed to solidify over-night.

Prior to electrophoresis of AFLP fragments, a pre-electrophoresis run at constant power (2000 V, 75 W, 100 mA) with a maximum temperature of 55 °C was carried out for 60 min. Selective Amplification PCR products were prepared for electrophoresis by adding 4 μ L loading buffer (98 % (v/v) formamide + 2 % (v/v) 0.5M EDTA pH 8 + xylene cyanol) to 4 μ L selective amplification product and denaturing DNA for 3 min at 94 °C. After denaturing, samples were maintained on ice until loading 6 μ L into a sharktooth comb. For molecular marker, 3 μ L of the 25 bp DNA ladder (Invitrogen-Life Technologies, Cat #10597-011, Carlsbad, CA, USA) was utilized. Electrophoresis was carried out with the same constant as the pre-electrophoresis run until xylene cyanol (slower dye) reach two-thirds down the length of the gel (approximately 3 hours and 30 min).

After each run, the glass plates in electrophoresis structure were carefully separated and the plate with bind silane containing the polyacrylamide gel was submitted to a silver staining method as described in Bassam *et al.*, 1991 (S 2). After staining, the polyacrylamide gels were allowed to dry before being photographed and analysed in detail for possible polymorphisms Adobe Photoshop 7.0. All bands appearing exclusively in heat stressed leaves or missing in heat stressed leaves were considered polymorphic. For further characterization, polymorphic bands were removed from the polyacrylamide gel using a small blade, mixed with 30 μ L of dH₂O, heated at 95 °C for 5 mins and immediately placed ice. Once centrifuged for 2 min at max speed to deposit the polyacrylamide, the suspended DNA was then utilized as template for PCR with equivalent specific selective primers using the same PCR program utilized for the selective amplification. Polymorphic PCR product were analyzed by 1 % (w/v) agarose gel electrophoresis, isolated, gel purified using the Pure Link Quick Gel Extraction Kit (Invitrogen-Life Technologies, Cat #K210012, Carlsbad, CA, USA) and sent for sequencing to STABVIDA (Monte de Caparica, Setúbal, 2825-182, Portugal).

2.6.3 - Genomic Analysis of identified AFLP Polymorphism

Polymorphic AFLP fragments were further analyzed in order to identify putative heat induced breakpoints in genomic DNA. Once removed from polyacrylamide gel and sequenced, the collected data was compared to the grapevine genome available at genoscope (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>) by BLAST-Like Alignment Tool (BLAT). Genomic sequences mapping within and flanking polymorphic fragments were utilized to design primers that permit the identification of putative heat induced breakpoints. These primers were then utilized for PCR on genomic DNA from control and heat stressed plants (HS-0 h, HS-24 h) where 20 μ L PCR mix contained 20 ng of genomic DNA, 1.5 mM MgCl₂, 20 pmol of each primer, 0.25 mM dNTP (deoxyribonucleotide triphosphates), and 1 U *Taq* polymerase. Reaction conditions were 25 cycles (95 °C 5 min, 25 cycles of 95 °C, 1 min; 65 °C, 1 min; and 72 °C, 1 min, and a final elongation step of 72 °C for 5 min). PCR products were separated by 1.5 % (w/v) agarose gel electrophoresis.

2.7 - RNA extraction and cDNA synthesis

For gene expression analysis, RNA was extracted as described in Chang *et al.*, 1993 (S 3) from young leaves (< 2 cm) collected from control canes or canes which had been exposed to heat stress (HS-0 h, and HS-24 h). After extraction, RNA integrity was analyzed by 1 % (w/v) Agarose gel electrophoresis, and its concentration and purity determined using Nanodrop.

Prior to cDNA synthesis, 5 μ g of total RNA was utilized for RNase free DNase digestion, using the kit RQ1 RNase-Free DNase (Promega, Cat # M6101, Madison, WI, USA). SuperScript III First-Strand Synthesis System for RT-PCR was used for first strand cDNA synthesis with random hexamers (dN₆) following manufacturer's instructions (Invitrogen-Life Technologies, Cat # 18080-051, Carlsbad, CA, USA)

2.8 - Quantitative Real Time (qRT-PCR)

BIO-RAD IQ5 Multicolor Real-Time PCR Detection System was utilized to analyze heat induced differences in transcript levels. PCR reactions were performed with the SsoFast EvaGreen Supermix (Bio-Rad Cat # 172-5203, Hercules, CA, USA) where each 20 μ L reaction mixture contained 1 μ L of cDNA (diluted 1:5 or 1:10) and 20 pmol of sequence specific primers and cycling conditions as described in Pereira *et al.*, 2009. The primers utilized can be divided in four groups, and are shown in Tables 2.3 and 2.8. Table 2.8 shows primers for control genes, *Actin2* and *nicotinamide adenine dinucleotide H (nadh) dehydrogenase subunit B* as well as genes related to DNA double-

strand break repair mechanism, *rad50* and *xrcc4*; and one AFLP polymorphic fragment. Table 2.3 shows primers for *Gret1* related sequences, LTR1, LTR2 and LTR3. All qRT-PCR experiments were repeated at least twice with three replicates per sample/primer combination in each experiment. All comparisons of expression levels were performed on identical cDNA dilutions.

Dissociation curves were observed to confirm correct single amplification products with expected denaturing temperatures. To ensure expected amplicon size, qRT-PCR products were separated by 1.5 % (w/v) agarose gel electrophoresis. For analysis of differential transcript levels, product threshold cycles (C_t) were normalized with mean C_t of control genes (*Actin2* and *NADH*) to calculate ΔC_t ($\Delta C_t = C_t$ of interest - mean *Actin2* or *NADH* C_t). $\Delta\Delta C_t$ was utilized to calculate fold change ($2^{-\Delta\Delta C_t}$) in expression levels between control and treated plants, where $\Delta\Delta C_t = \Delta C_{t,a} - \text{mean } \Delta C_{t,b}$, where a and b are being compared). Mean fold change ($2^{-\Delta\Delta C_t}$) \pm standard deviation and statistical analyses such as Student's t-test between control plants and plants submitted to heat stress were analyzed for all sequences using the program Microsoft® Excel 2007.

Table 2.8 – Primers utilized in the qRT-PCR to quantify *Vitis vinifera* expression.

Primers	Sequence
Actin2 - Forward	5`- GCTGGATTCTGGTGATGGTGTGAG
Actin2 - Reverse	5`- CAATGAGAGATGGCTGGAAGAGGAC
NADH dehydrogenase subunit B - Forward	5`- TGCAAGCATATGTTTCATGC
NADH dehydrogenase subunit B - Reverse	5`- CTGCTTCAGCTTCAGCCACT
Hsp90 - Forward	5`-AACTGAGAAAGAGATCAGTGATGATG
Hsp90 - Reverse	5`-GATAGTCCTCCCAGTCATTGGTCAG
Rad 50 – Forward	5`- GGAGAAGTTAAGGGATGAGCAA
Rad 50 – Reverse	5`- CCTCAACCAAACGATCAAGC
Xrcc4 – Forward	5`- CTGCACGCATCGTATCAAGT
Xrcc4 – Reverse	5`- CCGTCGGTGATAGAGAGGTG
AFLP Fragment - Forward	5`- GAATCCTTCATCAGTGGTTGTGAATG
AFLP Fragment - Reverse	5`- CGACAAAAGGATCACCCCTAAGATG

2.9 - Bioinformatic Analysis

The freely available program Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>) was utilized for primer design. Primers for *Gret1* related sequences were designed based on the complete *Gret1* (accession number AB111100.1). All sequences identified in this work were compared against the Grape Genome Browser (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>) by BLAT (8X) and BLAST. This grape genome database was also utilized to design gene specific primers, based on homology between gene sequences of model plant species and *Vitis vinifera*. Sequence comparisons were carried out using the free software Multalin, which uses a multiple sequence alignment with hierarchical clustering (<http://multalin.toulouse.inra.fr/multalin/>). All primer sequences and expected qRT-PCR amplification products were analyzed with the freely available Oligonucleotide Properties Calculator (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) to verify guanine/cytosine content and the expected annealing or dissociation temperatures, respectively.

3 – Results and Discussion

3.1 – Heat stress induced alterations on genomic structure

3.1.1 Heat stress treatments had no visible effects on vegetative development

Heat stressed plants (HS-0 h, and HS-24 h) maintained in controlled growing conditions continued to develop roots, leaves and inflorescences identically to control plants, as previously described (Pereira *et al.*, 2014). Regarding heat stress directly applied during reproductive growth, although leaves were slightly wilted after exposure to 42 °C for four hours (HS-0 h), they were indistinguishable from control plants after a 24 hour recovery period in controlled conditions (HS-24 h).

3.1.2- Integrity and concentration of genomic DNA

After DNA extraction, genomic DNA integrity, concentration and purity was checked for all samples utilizing by 1% (w/v) agarose gel electrophoresis and Nanodrop. Only samples with high quality genomic DNA were utilized for subsequent analysis, evident as single strong high molecular weight bands at the top of the gel and lack of smear demonstrating that the DNA is not fragmented, as shown in Figure 3.1.2. High quality genomic DNA was further supported by Nanodrop results, which showed concentrations ranging from 250 to 500 ng/μL and 260/280 ratios between 1.8 and 1.9, indicating lack of protein or phenolic compound contamination of samples.

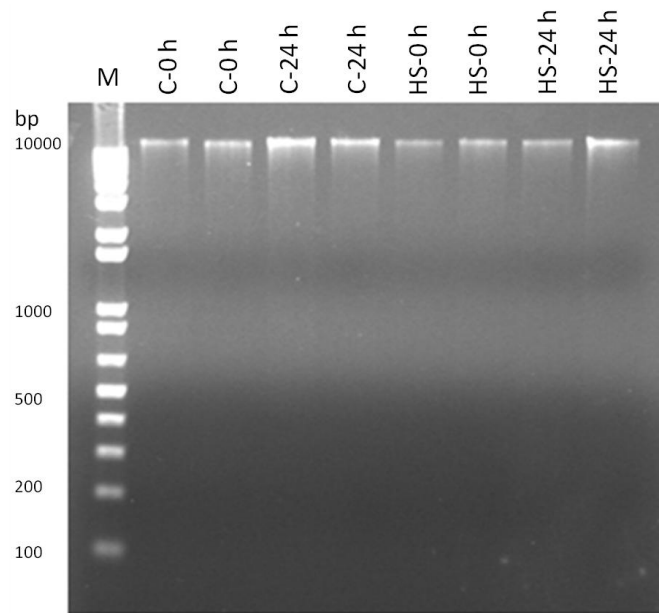


Figure 3.1.2 – Image of genomic DNA analyzed by 1 % Agarose gel electrophoresis with molecular weight marker (M, 1 Kb⁺ ladder with molecular weights shown in base-pairs) from biological replicates of control and heat stressed samples. Genomic DNA is shown from plants grown in controlled conditions and collected at 0 hours (C-0 h) or 24 hours (C-24 h), and heat stressed plants collected immediately after treatment (HS-0 h) or after a 24 hour recovery period (HS-24 h). High quality non-fragmented genomic DNA is evident as single sharp bands with high molecular weight for all samples.

3.1.3 - Heat induced alterations in the number of Gret1 related sequences

The number of *Gret1* related sequences was compared between control plants (C-0 h and C-24 h) and those exposed to heat stress and collected immediately after treatment (HS-0 h) and after a 24 hour recovery period (HS-24 h). Three *Gret1* related sequences mapping to the LTRs (LTR1, LTR2, and LTR3) as well as one sequence for the reverse transcriptase (*rt*) as shown in figure 3.1.3.1 were quantified.

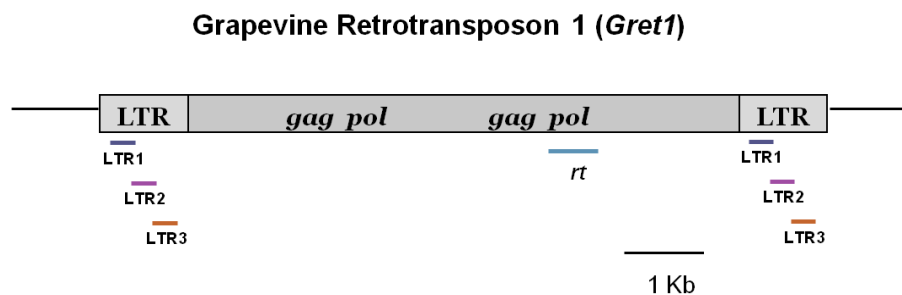


Figure 3.1.3.1 – *Gret1* complete sequence (10 420 bp) with 5`-LTR and 3`-LTR (824 bp each) flanking two open reading frames for the *gag* and *pol* genes. The three LTR (LTR1, LTR2 and LTR3) and reverse transcriptase sequences utilized for analysis are shown in relation to their location on the retroelement.

In order to quantify the number of *GretI* related sequences in each sample, regression lines were calculated using the Ct-values of the single SSR locus VVS2 for two biological replicates of genomic DNA isolated from controls and treatments. The eight regression lines and respective equation are demonstrated in Figure 3.1.3.3.

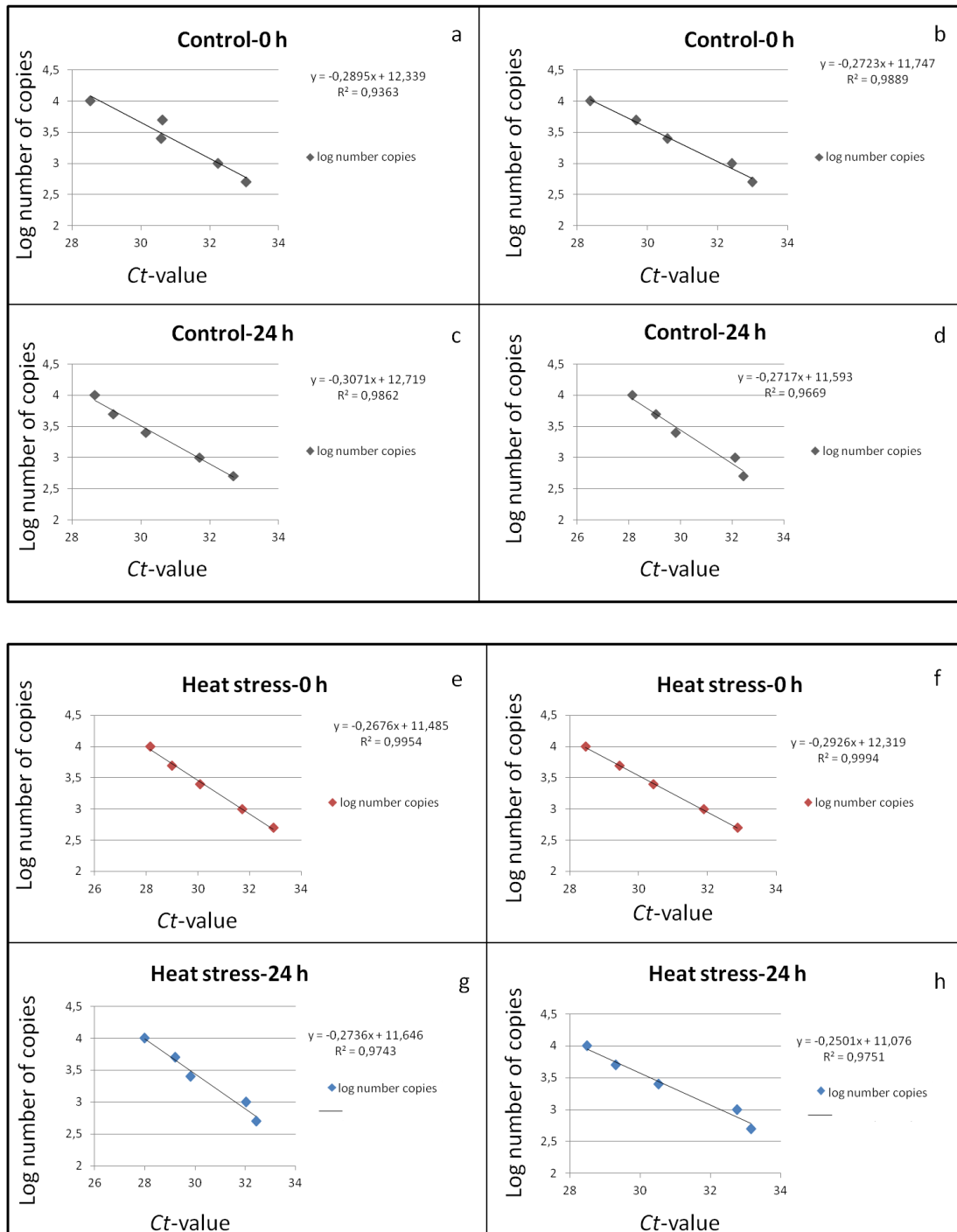


Figure 3.1.3.3 – Representation of regression lines calculated for all biological replicates correspondent to the Control-0 hours (a and b), Control-24 hours (c and d), Heat stress-0 hours (e and f) and Heat stress-24 hours (g and h). The coordinate axis (y) represents the log number of copies and the abscissa axis (x) the C_t -values from the SSR VVS2 dilutions.

The equation fitting each regression lines was then utilized to estimate the number of copies of each *Gret1* related sequence per haploid genome. All experiments were done for at least three technical replicates and three genomic dilutions, allowing for estimate of mean number of copies and associated standard deviation for each sequence. Means and standard deviations obtained for controls and heat stressed plants were compared utilizing Student's t-test, and results showed no significant differences between biological replicates allowing for pooling of results. Pooled results for controls and treatments are shown in Figure 3.1.3.4 and indicate a general decrease in the number of copies for all sequences which is significantly different at 24 hours after the heat stress (HS-24 h). In the case of LTR1, the mean \pm standard deviation of 71 ± 20 copies observed in DNA from control plants decreased to 61 ± 24 in heat stressed plants at 0 hours, and to 45 ± 12 in the heat stress-24 hours (Figure 3.1.3.4 a). For LTR2, the 228 ± 90 copies in controls diminished to 174 ± 84 in HS-0 h and to 94 ± 33 in HS-24 hours (Figure 3.1.3.4 b). As for LTR3, the control DNA contained 262 ± 115 copies, decreasing to 213 ± 88 copies in heat stress-0 hours, and 142 ± 43 after a 24 hours recovery period (Figure 3.1.3.4 c). Comparing the number of copies of the three *Gret1* LTR sequences, LTR2 and LTR3 were found to be two to three times more abundant in the genome than LTR1. The variation in number of copies between LTR sequences may be explained by the presence of non complete or fragmented *Gret1* related sequences in the *Vitis* genome. The number of copies of the *rt* locus showed the same tendency as the LTRs, with 1018 ± 523 copies in the controls and decreases to 847 ± 376 and 572 ± 558 for HS-0 h and HS-24 h, respectively (Figure 3.1.3.4 d).

Student's t-test showed that the difference in *Gret1* related sequences was not significantly different between DNA isolated from control plants and that isolated from heat stressed plants and collected immediately after treatment. This is evident as comparison between control and treatments resulting in p-values of: 0.22764, 0.12849, 0.19702 and 0.32652 for LTR1, LTR2, LTR3 and RT, respectively. On other hand, the number of copies of *Gret1* related sequences were highly significantly different between controls and heat stressed plants after 24 hours, with p-values of 0.00010, 0.00004, 0.00052 for LTR1, LTR2 and LTR3 respectively (Figure 3.1.3.4). Interestingly, this was not the case for the *reverse transcriptase* locus, where the decrease in number of copies at 24 hours is not significantly different ($p= 0.10360$). This lack of significance may be explained by the fact that *rt* is highly conserved between retrotransposons (Marn and Llore'ns, 2000) and therefore our primers may be amplifying this sequence from elements not related to *Gret1*. This is supported by the estimated thousands of copies specific to *rt* in comparison to the hundreds of *Gret1* LTR sequences. The difference between our heat induced results for LTR sequences and lack of significance for *rt* may indicate that heat has a stronger effect on *Gret1* than on other retrotransposons.

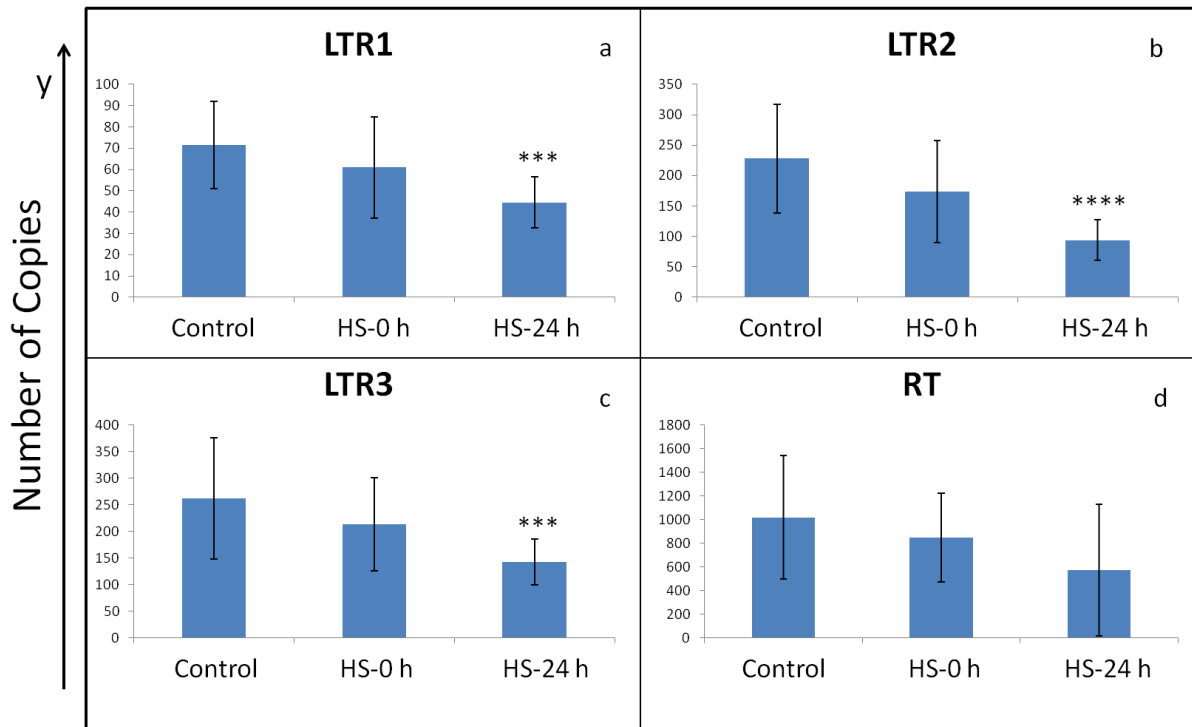


Figure 3.1.3.4 – Graphic representation of the number of copies from each of the four *Gret1* related sequences in controls and heat treated plants (HS-0 h and HS-24 h). Number of copies of LTR1 (a), LTR2 (b) LTR3 (c) and *reverse transcriptase* (RT) (d). The coordinate axis represents the number of copies per haploid genome and average numbers of copies from all respective samples/triplicates \pm standard deviation (black line, above each bar) are shown. Asterisks represent level of significance as estimated by Student’s t-test (Student’s t-test, where ***, $p < 0,001$).

The results from the qRT-PCR demonstrate a significant decrease in the number of copies of *Gret1* related sequences in heat stressed plants after a 24 hour recovery period. Considering that retrotransposons are known for their “copy-and-paste” propagation mechanism, one may expect heat induced activation of *Gret1* to lead to an increase of copies in the genome. Importantly, the experiments in this work were carried out on leaves, where cells are generally non-cycling (lack of mitotic divisions) and are mostly in interphase. One putative explanation for the loss of *Gret1* copies is that heat induced DNA damage such as double strand breaks, which were not properly repaired. In previous studies it was observed that *Gret1* accumulates in the genome in tandem repeats (Pereira *et al.*, 2005) which can explain the abundant loss of copies during DNA repair. In this same study it was demonstrated that *Gret1* maps to gene rich regions of the genome. This may indicate that heat induced loss of *Gret1* related sequences can putatively affect gene function.

3.1.4 –IRAP and REMAP did not identify heat induced polymorphisms

REMAP and IRAP based on *Gret1* sequences were used to investigate possible genomic alterations flanking *Gret1* genomic regions and results are shown in Figure 3.1.4. These techniques did not identify polymorphisms, evident as identical banding patterns between controls and heat stressed

plants. Regarding IRAP with primer *Gret1LTR-reverse*, only 3 fragments with approximately 850 bp, 650 bp and 500 bp were amplified in all samples (Figure 3.1.4 a). With the REMAP was observed a more complex pattern, still no differences were present in the two combinations used. REMAP *Gret1LTR-reverse/Microsat-GA* resulted in the most complex band pattern from the three combinations used, 9 bands with molecular weights between 100 and 700 base pairs in all samples (Figure 3.1.4 b). REMAP *Gret1LTR-forward/Microsat-CT* also did not identify polymorphism, evident as identical seven amplification products with molecular weights between 100 and 2000 bp for all samples (Figure 3.1.4 c). The banding patterns observed were compared with previous REMAP and IRAP results also realized in Touriga Nacional (Pereira *et al.*, 2005). The REMAP banding patterns for primer combination *Gret1LTR-forward/Microsat-CT* were identical to previous results and *Gret1LTR-reverse/Microsat-GA* showed similar pattern considering that only the major bands were observed. Interestingly, IRAP *Gret1LTR-reverse* produced identical banding pattern as observed in published for *Gret1LTR-forward/Gret1LTR-reverse*, demonstrating that the *Gret1LTR-reverse* primer is responsible for all amplification products. Considering the lack of complexity of banding patterns obtained with REMAP and IRAP and the *V. vinifera* genome size of 508 Mbp (Jaillon *et al.*, 2007 and Velasco *et al.*, 2007), it is not surprising that these techniques did not identify the DNA breakpoints associated with the *Gret1* loss identified in the qRT-PCR.

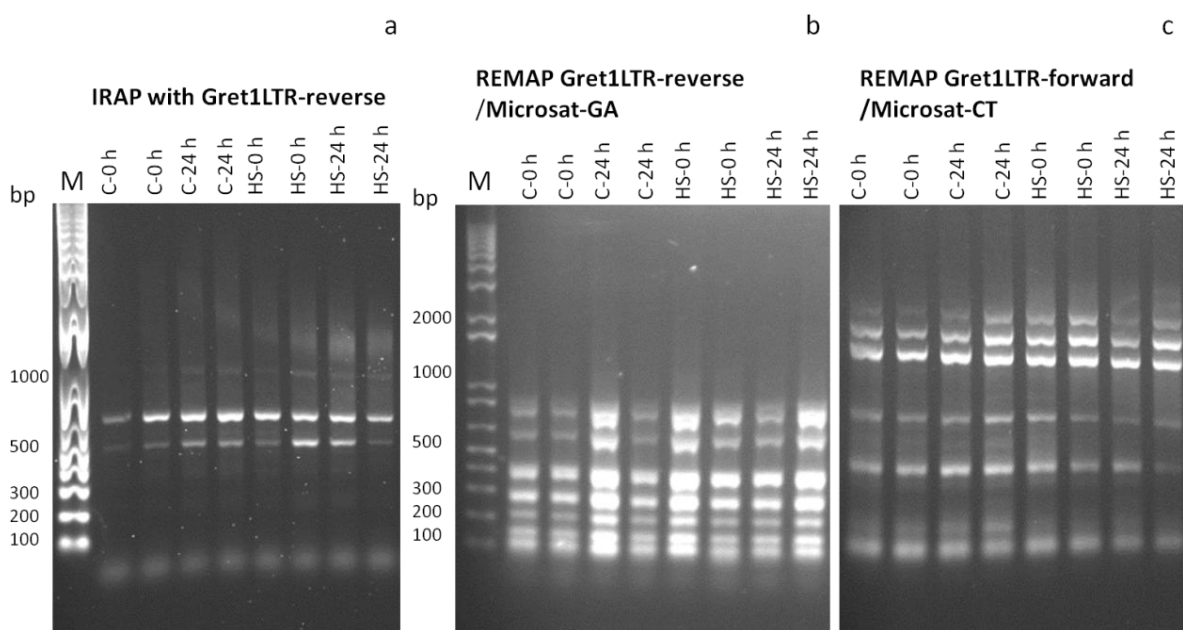


Figure 3.1.4 – Products from IRAP and REMAP analyzed in 1 % (w/v) Agarose gel electrophoresis. IRAP using the primer *Gret1LTR-reverse* (a). REMAP using the primer combination *Gret1LTR-reverse/Microsat-GA* (b), and *Gret1LTR-forward/Microsat-CT* (c). Molecular weight marker (M, 1 Kb⁺ ladder), control-0 hours (C-0 h), control-24 hours (C-24 h), heat stress-0 hours (HS-0 h), heat stress-24 hours (HS-24 h).

3.1.5 – Southern Blotting did not detect heat induced differences in DNA methylation

Southern blotting technique was utilized in order to uncover putative differences in DNA methylation of *Gret1* related genomic sequences. For detection the LTR2 was chosen as probe in expense to the other LTRs sequences, for its length and positioning the LTR. As shown in Figure 3.1.5, a single low intensity signal was obtained for a high molecular weight DNA fragment at the top of the membrane. The lack of clear results was not due to the technique having failed, as the molecular weight marker is clearly visible. However, the difference in signal intensity between the right and left control λ *HindIII* molecular weight marker clearly indicates that probe signal was not homogeneous, with the left portion of the membrane being of better quality. Accordingly, the lack of signal in the right portion of the membrane renders it impossible to analyze potential differences in the methylation status of *Gret1* related sequences. In comparison to previous southern blotting results of *Gret1* related sequences (Pereira *et al.*, 2005), in this work we did not detect a strong smear observed with the *rt* probe. This may be explained by the fact that the *rt* sequence is represented in many more copies in the genome than LTR2 sequence as demonstrated above in Figure 3.1.3.4.

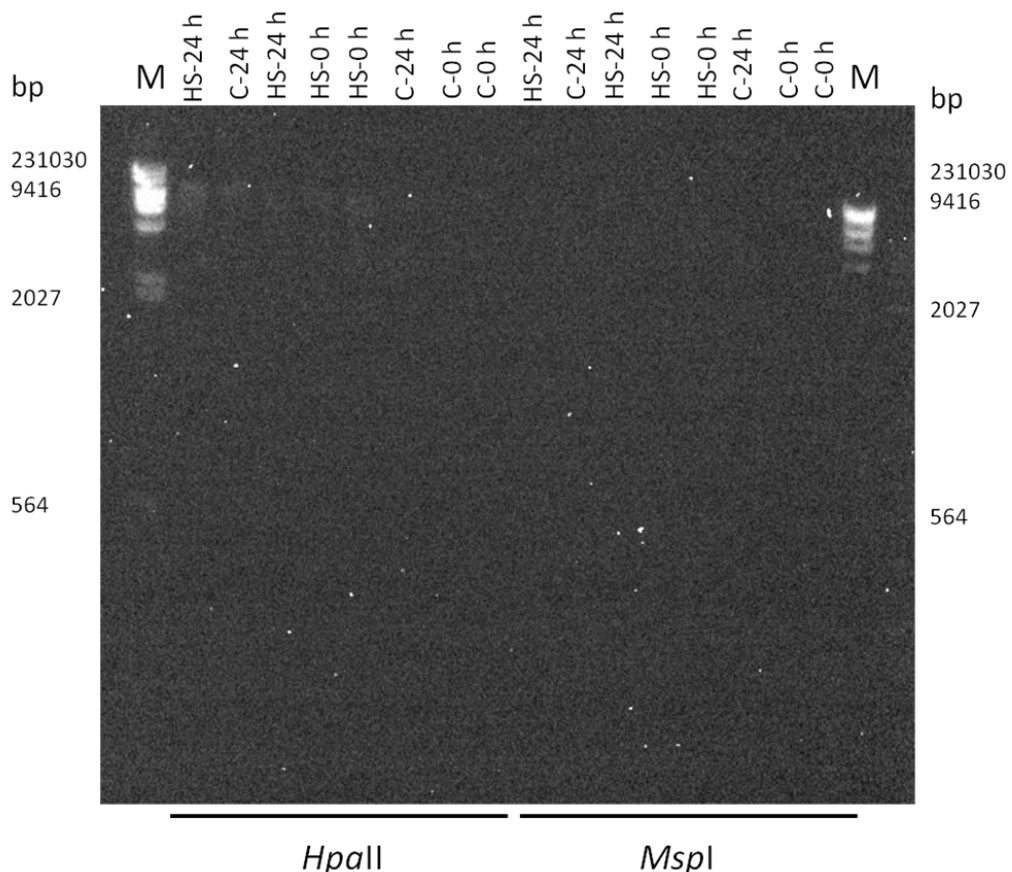


Figure 3.1.5 - Image of Southern Blot probed with LTR2 sequence and genomic DNA digested with *HpaII* (sensitive to methylation) and *MspI* (non sensitive to methylation) with molecular weight marker (M, λ *HindIII*). Samples are shown at the top of the gel, and consist of HS-24 h, C-24 h, HS-24 h, HS-0 h, C-24 h, C-0 h and C-0 h.

3.1.6 – Amplified Fragment Length Polymorphism (AFLP) identified one DNA polymorphism between heat stressed and control plants

In order to identify putative heat induced genomic modifications, the genome wide PCR based molecular technique AFLP was realized. Prior to the final PCR (selective amplifications), PCR pre-amplification PCRs were performed and observed by 1% (w/v) agarose gel electrophoresis. As shown in Figure 3.1.6.1, pre-amplification PCRs resulted in a smear between the 100 to 500 bp containing genomic DNA fragments flanked by the *EcoRI* and *MseI* adapters.

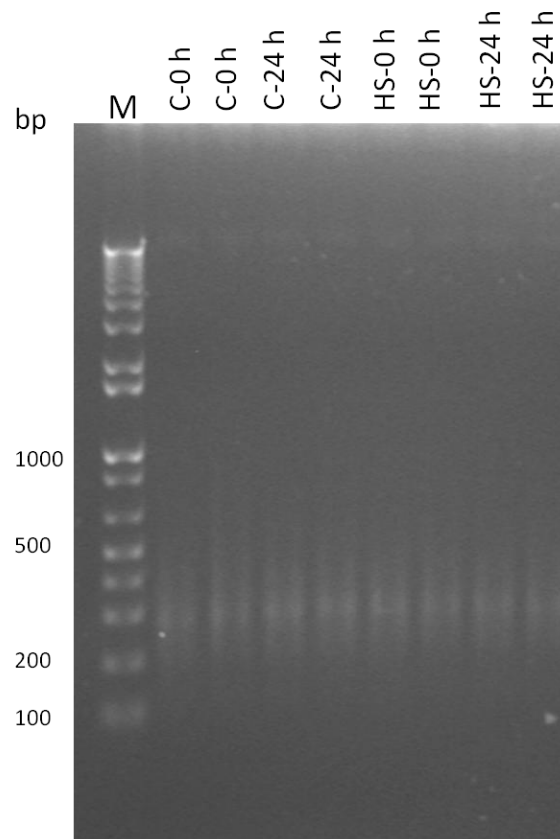


Figure 3.1.6.1 – AFLP pre-amplification products analyzed in a 1 % (w/v) Agarose gel electrophoresis show a smear between 200 and 500 bp. As shown on top of the gel, samples include molecular weight marker (M, 1 Kb⁺ ladder), and pre-amplification products from controls (C-0 h and C-24 h) and both heat treatments (HS-0 h and HS-24 h).

Selective amplifications were performed with five primer pairs (Eco-TA+Mse-CAA, Eco-TC+Mse-CTT, Eco-TG+Mse-CAA, Eco-CT+Mse-CTT and Eco-TT+Mse-CTG) and banding patterns observed by denaturing polyacrylamide gel electrophoresis (data not shown). Although the banding profiles obtained by AFLP were highly complex, only one primer combination (Eco-TC+Mse-CTT) resulted in the identification of a polymorphic DNA fragment between heat stressed and control plants. This pair of primers resulted in a polymorphism corresponding to the absence of an amplification product of approximately 260 bp in one biological replicate HS-0 h and one HS-24 h plant (Figure 3.1.6.2). Regarding the nature of the polymorphism, it is likely to map to a repetitive

region due the intensity and thickness of the corresponding AFLP product. The presence of two thick bands with approximately 285 bp, with the same pattern of intensity, especially for the HS-0 h and HS-24 h, further supports the idea of being a repetitive region. In order to characterize the genomic sequences absent in both heat treatments, the corresponding fragments from C-0 h and C-24 h were isolated from the gel, re-amplified and sequenced as described below.

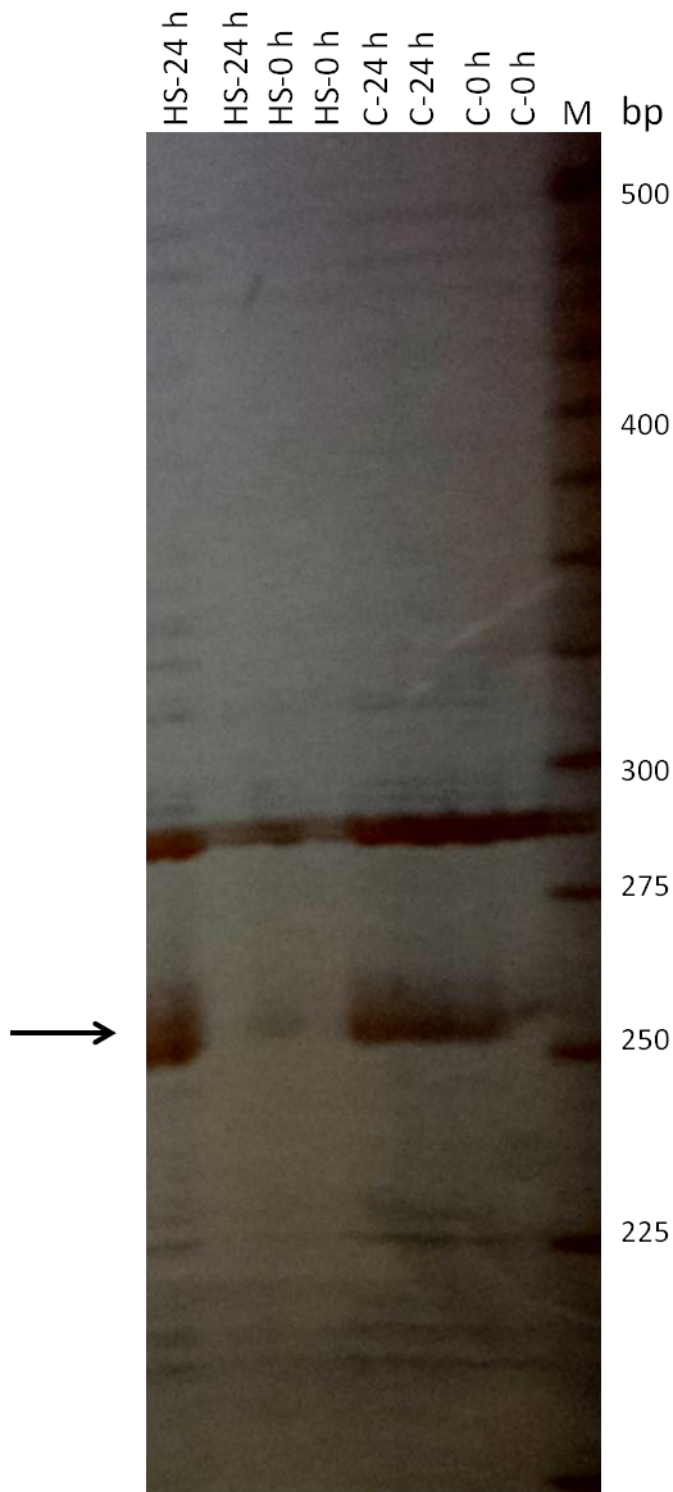


Figure 3.1.6.2 – Denaturing polyacrylamide gel electrophoresis of AFLP products obtained with Eco-TC/Mse-CTT primers. Samples are shown on top of the gel, and include heat stress-24 hours (HS-24 h), heat stress-0 hours (HS-0 h), control-24 hours (C-24 h), control-0 hours (C-0 h), and molecular weight marker (M, 25 bp ladder). The black arrow indicates a heat induced polymorphism, evident as absence of a fragment with approximately 260 bp in one HS-0 h and one HS-24 h sample.

3.1.7 - Heat induced AFLP polymorphism

The AFLP polymorphic fragment was further analyzed by isolating the corresponding DNA from the control samples (C-0 h and C-24 h), re-amplifying, and sequencing with the Eco-TC/Mse-CTT specific primers utilized for selective amplification. High quality sequence data was aligned on multalin (<http://multalin.toulouse.inra.fr/multalin/>) for construction of a 264 bp consensus sequence. As demonstrated in Figure 3.1.7.1, this sequence was of the expected molecular weight and contained both *EcoRI* and *MseI* adaptor sequences at each extremity as expected.

```
tgatgagtcctgag TAACTT TTCGAGGAAT CCTTCATCAG TGGTTGTGAA TGACTGATT
TTTCAATCTT TTCGACCTTG GTTCCGTAGG AGCAAGTCAG AAAGATGAGA ATAGAACCAT
CTAATTTGAT TCGTTCCTCAA TAGCCATGAG ATGATCATCT TAGGGTGATC CTTTTGTCGA
CGGATGCTCC TAATANACTC GTAGTCTCTG AAGGATGAGA ACCAACTATG TAGCATCTAC
ATCGAGAATT ggtacgcagcaagt
```

Figure 3.1.7.1 – Consensus sequence of gel isolated AFLP polymorphic band with the *EcoRI* and *MseI* adaptors in extremities (in lowercase).

The consensus sequence demonstrated in Figure 3.1.7.1 (without the *EcoRI* and *MseI* adapters) was utilized for BLAST-Like Alignment Tool (BLAT) with the grapevine genome (8x) available at genoscope (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>). As expected from the thickness and intensity of the AFLP band, the BLAT results showed various entries with high homology on the genome, reinforcing the idea of being a repetitive sequence (Figure 3.1.7.2). The first three entries map the AFLP-Fragment sequence on chromosome 2. In all three cases approximately 500 bp downstream of the *nicotinamide adenine dinucleotide H (nadh) dehydrogenase subunit B*. To further explore the genomic region of these three entries, various primers were designed based on homology within the AFLP fragment sequence or flanking genomic DNA, as shown in Table 3.1.7 and Figure 3.1.7.3 a. For this, 1 Kb sequences isolated from the first three BLAT entries were aligned on multalin (<http://multalin.toulouse.inra.fr/multalin/>). Due to the high level of sequence identity between the first two sequences, it was not possible to design specific primers to each one. Therefore, three sets of primers were designed in order to amplify genomic regions upstream or downstream of the AFLP – Fragment from two first entries of the BLAT (AFLP -1and2), the third entry (AFLP-3), or all three entries (AFLP –all) from chromosome 2.

	SCORE	START	END	QSIZE	IDENTITY	CHROM	STRAND	START	END	TSIZE	LENGTH
Sequence 1 -	139	1	153	153	96.1%	chr2	+	14261332	14261486	17603400	155
Sequence 2 -	136	1	153	153	95.4%	chr2	+	14290411	14290565	17603400	155
Sequence 3 -	136	1	153	153	95.4%	chr2	+	14466605	14466759	17603400	155
	108	36	153	153	96.7%	chr1	-	4704836	4704955	15630816	120
	98	23	153	153	93.9%	chr19	+	12716686	12716820	14071813	135
	76	1	101	153	93.2%	chrUn_random	-	45452041	45452142	154883714	102
	75	75	153	153	98.8%	chr5	+	11324646	11324726	23428299	81
	57	80	153	153	89.2%	chr1	-	12562617	12562691	15630816	75
	47	100	152	153	94.4%	chr5	-	17004104	17004156	23428299	53

Figure 3.1.7.2 –BLAT results showing polymorphic AFLP sequence against the grapevine genome (8x) showing score, the start and end of the homology with genomic sequence, percentage identity, the location on the genome (chromosome, strand, and nucleotides) and length of homology. The three entries used for genomic analysis are highlighted in red (Sequence 1, sequence 2 and sequence 3).

Table 3.1.7 – Primers designed for AFLP polymorphic fragment and flanking genomic regions.

Primers	Sequence
AFLP Fragment - Forward	5`-GAATCCTTCATCAGTGGTTGTGAATG
AFLP Fragment - Reverse	5`-CGACAAAAGGATCACCTAAGATG
AFLP -1and2 - Forward	5`-GATGTCATTCTAAATCAATGACATCCTTG
AFLP -1and2 - Reverse	5`-ATAGACCAACCTCAATGATCGTAGAC
AFLP -3 - Forward	5`-CCTGTATAATCCTGCATAATCTCG
AFLP -3 - Reverse	5`-GGGGAGAGATCAAGCTTCAAG
AFLP -all - Forward	5`-TTACCTTCCTCGCGTATGTATAAG
AFLP -all - Reverse	5`-CCTACGAAGGATCAAATAATGGC

Primers designed based on the three entries on chromosome 2 and within the AFLP polymorphic fragment were utilized for PCR in order to identify heat induced genomic modifications as shown in Figure 3.1.7.3 a. The amplification products of all combination of primers amplifying within the AFLP fragment and upstream or downstream genomic regions were identical for all samples (Figure 3.1.7.3 b and c). PCRs with primers amplifying between the flanking genomic region of all three sequences and within the AFLP fragment result in a product with the expected size of 200 bp, as shown on the right gel of Figure 3.1.7.3 b and c. Primer combination AFLP-Fragment-Forward/AFLF-all-Reverse also amplified a second more tenous fragment with a molecular weight of approximately 100 bp. Primer combinations AFLP1and2-Forward/AFLP-Fragment-Reverse and AFLP-3-Forward/AFLP-Fragment-Reverse resulted in duplet bands with molecular weights of approximately 500 bp, as shown in the left and center gel of Figure 3.1.7.3 b.. In contrast, the combinations AFLP-Fragment-Forward/AFLP1and2-Reverse and AFLP-Fragment-Forward/AFLP-3-Reverse had a unique strong band with the expected molecular weight of approximately 500 bp, as

shown in the left and center gel of Figure 3.1.7.3 c. In conclusion, these PCRs did not identify a heat induced alteration in the genomic DNA flanking the polymorphism observed in the AFLP. This could be explained by the fact that this sequence is repeated many times in the *V. vinifera* genome, as shown by BLAT. On the other hand, the heat induced polymorphic band may have resulted from a small insertion/deletion mutation within the AFLP fragment that affected the recognition site of the restriction enzymes utilized for AFLP. Future work involving PCRs and restriction digested genomic DNA will undoubtedly shed insight into the genomic modification identified by AFLP.

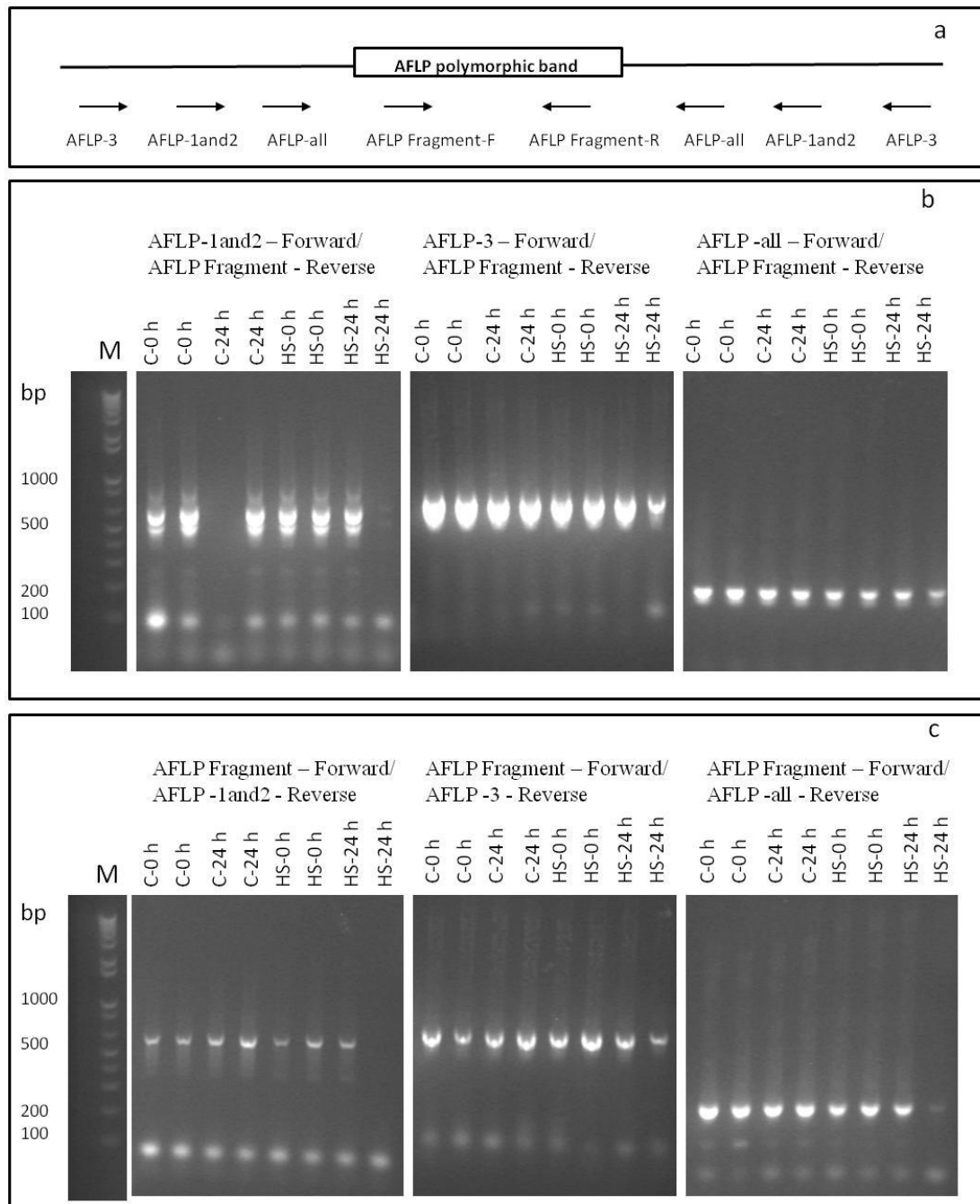


Figure 3.1.7.3 – Representation of primers and respective location within or flanking the AFLP –Fragment utilized to examine genomic region of AFLP identified polymorphism (a). 1.5 % (w/v) agarose gel electrophoresis with molecular weight marker (M, 1 Kb⁺ ladder) and samples C-0 h, C-24 h, HS-0 h and HS-24 h (b and c). PCR products obtained with primer sets AFLP-1and2-Forward/AFLP-Fragment-Reverse (left gel), AFLP-3-Forward/AFLP-Fragment-Reverse (center gel), and AFLP-all-Forward/AFLP-Fragment-Reverse (right gel) (b). PCR products obtained with primer sets AFLP-Fragment-Forward/AFLP-1and2-Reverse (left gel), AFLP-Fragment-Forward/AFLP-3-Reverse (center gel), and AFLP-Fragment-Forward/AFLP-all-Reverse (right gel) (c). 34

3.2 – Heat stress induced alterations on transcription

3.2.1 – Integrity and concentration of RNA

RNA extractions were analyzed for RNA integrity, concentration and purity by 1% (w/v) agarose gel electrophoresis and Nanodrop. For transcription analyses only RNA with no signs of degradation were used for cDNA synthesis, as observed in Figure 3.2.1 with a two strong evident bands between the 500 and 1000 bp representing the ribosomal RNAs. The Nanodrop results confirmed the good quality of the RNA, with purity ratios (260/280) between 2.0 and 2.1, confirming no signs of protein contamination, and concentration from 1000 to 2000 ng/ μ L.

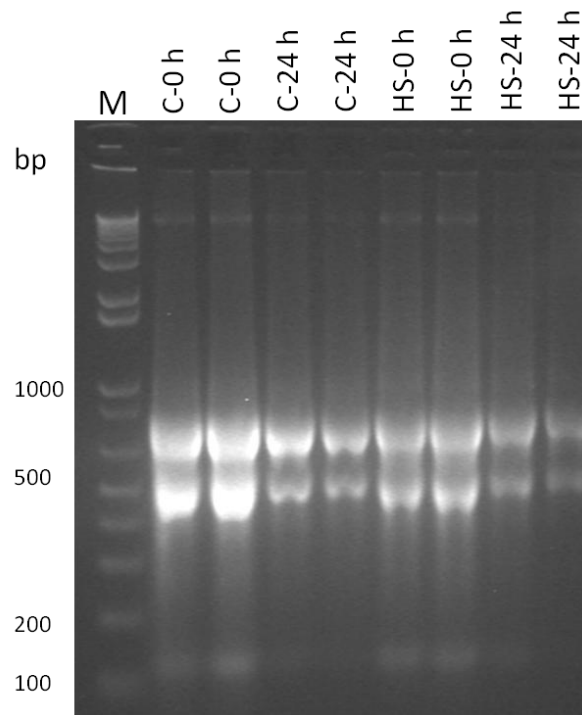


Figure 3.2.1 – Image of 1 % (w/v) agarose gel electrophoresis of RNA, with molecular weight marker (M, 1 Kb⁺ ladder) and samples control-0 hours (C-0 h), control-24 hours (C-24 h), heat stress-0 hours (HS-0 h), heat stress-24 hours (HS-24 h). Two ribosomal RNA bands with the expected molecular weights of 500 and 1000 bp can be seen.

3.2.2 – Effects on transcription caused by heat stress

Heat stress effects were analyzed at the transcriptomic level by quantitative real time PCR (qRT-PCR). For this, it was necessary to choose reference genes with expression levels that were independent of heat stress such as *Actin2*, which is commonly utilized as a control gene in transcription analyses (Pereira *et al.*, 2009). Initially, the transcript levels of a gene encoding the *nadh dehydrogenase subunit B* (NADH) were analyzed due to its proximity to the identified AFLP – Fragment. As shown in Figure 3.2.2, qRT-PCR results showed that NADH transcript level is not

altered by heat stress, permitting for its use as a reference gene, as reported in other studies (Zhao *et al.*, 2014). Primers specific for the gene encoding for the HSP90 protein were utilized to confirm that the plants were responding to the heat stress. Regrettably, it was not possible to analyze the fold changes involved in heat induced levels of expression of HSP90 due to dissociation temperatures between controls and heat-stress-24 hours being different from those of heat stress-0 h hours. This result can be explained by alternative splicing and is supported by a slight reduction in the molecular weight of the qRT-PCR product from HS-0 h in comparison to controls or HS-24 h, as shown in Figure 3.2.2. Gel electrophoresis established heat stress responsive up-regulation of *hsp90*, evident as a much stronger signal at HS-0 h in comparison to control (Figure 3.2.2). Interestingly, there appeared to be a significant decrease in HS-24 h, likely corresponding to previously described repression of HSP90 suffered during the recovery time (Banilas *et al.*, 2011).

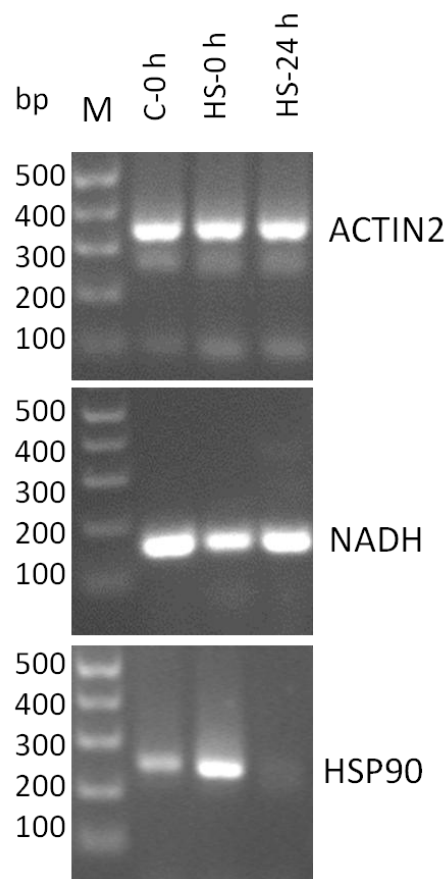


Figure 3.2.2 – Results of 1.5 % (w/v) agarose gel electrophoresis of qRT-PCR products of Actin2, NADH and HSP90, genes for control-0 hours (C-0 h) and both heat treatments (HS-0 h and HS-24 h). The correspondent size of each band is demonstrated by the molecular weight maker, 1 Kb⁺ ladder (M).

3.2.3- Heat stress affects transcription of non gene coding regions

Quantitative RT-PCR was utilized to analyze the effects of heat on the expression of *Gret1* related sequences (LTR1, LTR2 and LTR3). As previously reviewed in the introduction, many studies showed a heat induced increase in the expression of retroelements (Salazar *et al.*, 2007; Pecinka *et al.*, 2010; Tittel-Elmer *et al.*, 2010; Cavrak *et al.*, 2014). Here, our results also indicated that *Gret1* had higher levels of expression in samples collected immediately after heat stress, as shown in Figure 3.2.3.1. Due to the low level of expression evident as high C_t s as well as all LTR sequences resulting in multiple dissociation curves (with disparate melting temperatures), it was impossible to obtain fold changes in expression and analyze the data statistically. However, visualization of LTR1, LTR2 and LTR3 results by 1.5 % (w/v) agarose gel electrophoresis established upregulation of all three LTR sequences with the expected sizes (LTR1 – 177 bp, LTR2 – 237 bp and LTR3 – 152 bp) as well as less intense secondary amplification products.

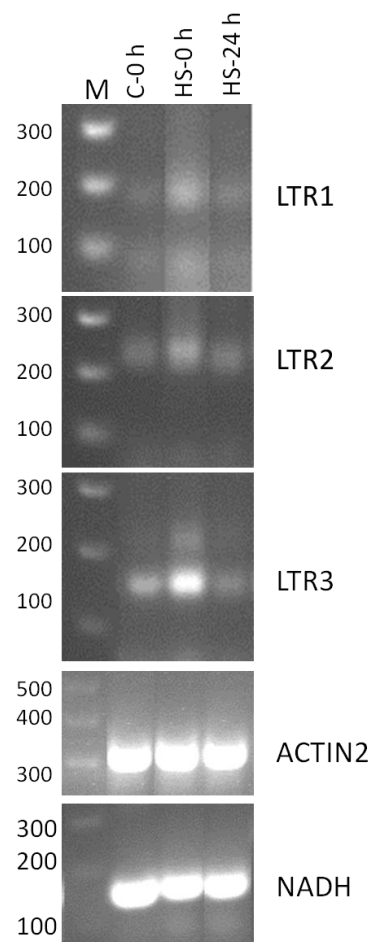


Figure 3.2.3.1 - 1.5 % (w/v) agarose gel electrophoresis of qRT-PCR results of LTR1, LTR2 and LTR3 for control (C-0 h) and both heat treatments (HS-0 h and HS-24 h) show up-regulation of *Gret1* related sequences at HS-0 h. The expected molecular weight of each band is demonstrated by the molecular weight maker, 1 Kb⁺ ladder (M). Both control genes (Actin2 and NADH) are shown as loading controls.

The expression of the heat induced polymorphic AFLP – Fragment was also analyzed by qRT-PCR with primers shown in Table 3.1.7. As represented in Figure 3.2.3.2, there was no difference in the transcript level of this genomic sequence between controls and heat stressed plants, with heat stressed plants having a 1.2 fold change in relation to controls.

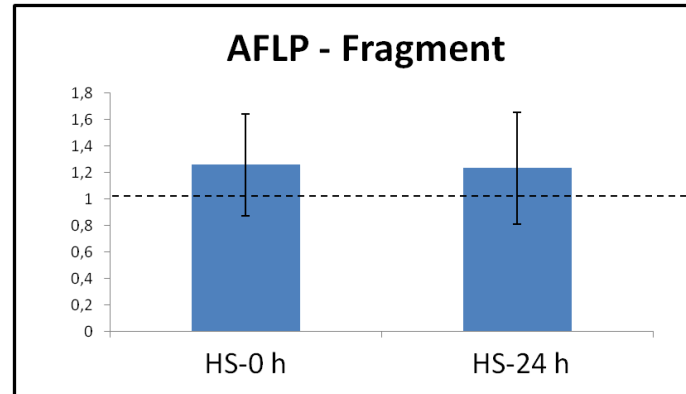


Figure 3.2.3.2 – Graphic representation of mean fold changes \pm standard deviations of AFLP – Fragment transcript level from heat stress-0 hours (HS-0 h) and heat stress-24 hours (HS- 24h) in relation to controls.

3.2.4- Heat stress impairs expression of genes involved in DNA double strand break repair

One putative biological explanation to justify the loss of *Gret1* related sequences in the genomic DNA of heat stressed leaves collected 24 h after a recovery period HS-24 h is that heat induced double strand breaks were not properly repaired in this non-cycling leaf tissue. This is supported by several studies showing that heat stress can cause DNA damage and DSBs (Kaneko *et al.*, 2005; Hunt *et al.*, 2007; Takahashi *et al.*, 2008; Laszlo and Fleischer, 2009b). This type of DSBs damage is usually corrected by a non-homologous end joining (NHEJ) pathway in cells with no mitotic activity (reviewed in Yoshiyama *et al.*, 2013). In order to test this hypothesis, qRT PCR was utilized to test variation in the transcript levels of two genes involved in this pathway, including *rad50* which belongs to the complex that identifies the DSBs (Rupnik *et al.*, 2010) and *xrcc4*, an important factor that recruits the Lig4 for DSBs repair (Grawunder *et al.*, 1997; Critchlow *et al.*, 1997). The results for the *rad50* showed an extreme down-regulation of this gene in HS-0 h plants, where product could not be detected in sufficient amount to calculate fold change. After a 24 hour recovery period (HS-24 h), the transcript level of this gene was found to be identical to control, with a mean fold change \pm standard deviation of 0.88 ± 0.18 in relation to controls (Figure 3.2.4.1 a). Similar heat induced regulation of *xrcc4* transcript level was observed, with no detected gene product at HS- 0 h returning to a not significant mean fold change of 0.68 ± 0.16 for HS-24 h in relation to control (Figure 3.2.4.1 b). These results are corroborated by observation of *rad50* and *xrcc4* amplified products analyzed by 1.5 % (w/v) agarose gel electrophoresis, where down regulation of qRT-PCR products of the expected size are evident at HS-0 h as shown in Figure 3.2.4.2. These results may be a

putative explanation for the observed loss of *Gret1* related sequences in genomic DNA. In this hypothesis, DSBs are created during heat stress when there is a down regulation in the expression of genes related to DSBs identification and repair (HS-0 h). Although after a 24 hour recovery period (HS-24 h) the expression of genes involved in DSBs identification and repair are identical to those of controls, the down regulation of these genes upon heat stress together with the intrinsic mutagenic capacity of the NHEJ pathway, could result in loss of sequences during the reparation process (reviewed in Yoshiyama *et al.*, 2013).

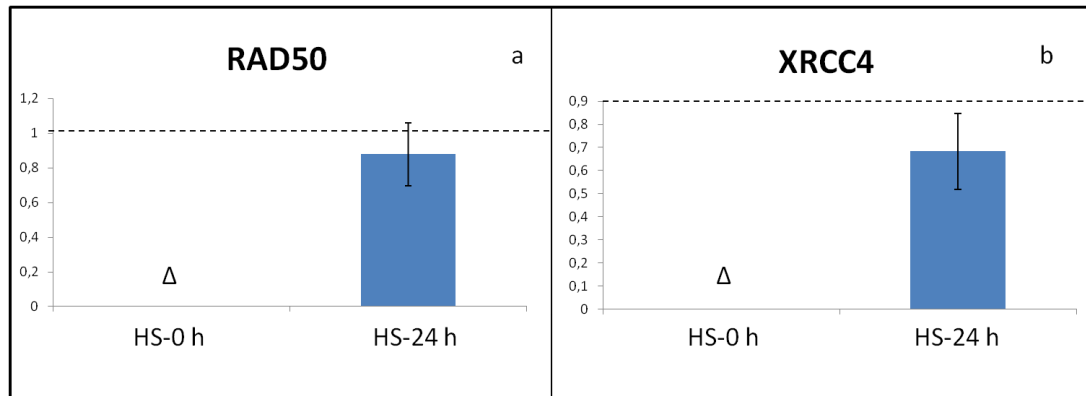


Figure 3.2.4.1 – Graphic representation of the mean fold change and respective standard deviations in transcript levels between control and heat stressed plants immediately after treatment (HS-0 h) and after a 24 hour recovery period (HS- 24h) for RAD50 (a) and XRCC4 (b). The small triangle (Δ) represents transcript was not detected in sufficient amount to calculate fold changes.

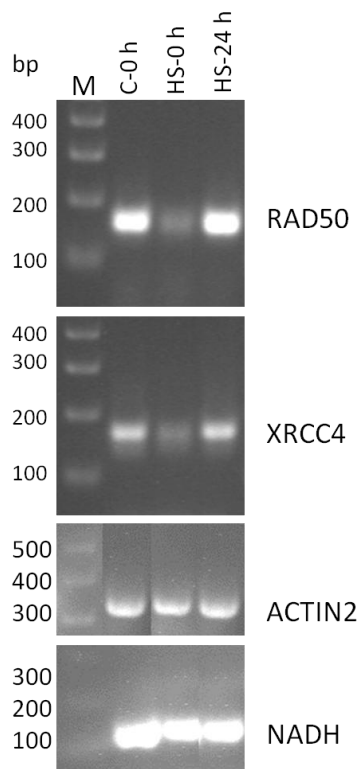


Figure 3.2.4.2 - Image of the qRT-PCR products for the primers RAD50 and XRCC4 and reference genes (Actin2 and NADH) on 1.5% (w/v) agarose gel electrophoresis from controls and heat stressed plants (HS-0 h and HS-24 h). Molecular weights of PCR products are demonstrated by the molecular weight maker, 1 Kb⁺ ladder (M).

4 - Conclusions

This thesis provides evidence that heat stress induces genomic alterations in retrotransposon sequences and affects transcription levels in *Vitis vinifera*. Interestingly, although retrotransposons propagate by “copy-and-paste” mechanism, which tends to increase their number of copies in the host genome, the opposite was observed for *Gret1* where heat stress caused a significant decrease in copy number. Results from the *rt* and LTRs sequences demonstrate that this loss of copies is likely specific to *Gret1* and likely in genomic regions where *Gret1* related sequences are fragmented and present in tandem repeats. One putative biological explanation for these results is that heat induces DNA damage such as double strand DNA breaks and/or errors during their reparation.

A number of genome wide approaches were utilized in order to try to identify the genomic regions modified by heat stress. Amplified Fragment Length Polymorphism identified one heat induced polymorphism mapping to a repetitive genomic region flanking a house-keeping gene. Due to its repetitiveness in the genome, it was not possible to identify the precise genomic location of the alteration which caused the polymorphism. However, future genomic analyses involving all the sequences with homology to this polymorphism in conjunction with *Gret1* related sequences will be carried out. Furthermore, analysis of possible mutations involving small insertion/deletions may be important for reaching the final objective of developing molecular markers specific for heat induced alterations.

Besides genomic alterations, heat can also cause variations at the transcriptional level. Using a quantitative Real-Time PCR, an increase in the expression of LTR sequences from *Gret1* subsequent to the heat stress was shown. Furthermore, analyses regarding the expression of genes associated with identification and repair of DSBs reinforced our putative explanation for the loss of *Gret1* copies. In cells with no mitotic activity, as is the case for leaves, the primary repair mechanism of DSBs is the mutagenic-induced non-homologous end joining (NHEJ) pathway, which can lead to errors during repair and in turn deletion of genomic regions. Interestingly, a down regulation of two genes important

for NHEJ was observed immediately following heat stress, with transcription levels returning to normal after a 24 hour recovery period. A bioinformatics analysis in *Arabidopsis* has shown that the regulation of genes involved in heat stress response is greater in complexity in comparison to other abiotic stresses responsive genes, such as those responsive to cold, salt, wounding, drought, and oxidative stress conditions (Sun *et al.*, 2010). Future studies involving heat induced DNA damage such as DSBs in non gene coding repetitive genomic regions and transcriptional analysis of relevant genes will undoubtedly aid in providing essential tools in the ever changing environment.

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Supplementary data

S 1- DNA extraction protocol, adapted from Thomas *et al.* 1993

1. Approximately 1 g of vegetative material in liquid nitrogen was ground to a fine powder with a mortar and pestle;
2. Powdered vegetable material was transferred to a tube containing 300 μL of extraction buffer (0.35 M sorbitol, 0.1 M Tris, 5 mM EDTA, 1 % (w/v) PVP), 3.8 g/L of sodium bisulfate and 1 % (v/v) of 2-mercaptoethanol and tube was placed on ice;
3. 300 μL of lysis buffer (1 M Tris, 0.25 M EDTA, 5 M NaCl, 20 g CTAB, at pH 7) and 120 μL of 5 % (w/v) sarkosyl were added;
4. Samples were incubated at 65 $^{\circ}\text{C}$ for 15 min;
5. 600 μL of chloroform/isoamyl alcohol (24:1) was added to each tube and vortexed until emulsion was formed;
6. Samples were centrifuged at 13 000 rpm for 15 min at room temperature;
7. Supernatant was collected and transferred to a new tube and 400 μL of isopropanol at 0 $^{\circ}\text{C}$ was added and gently mixed;
8. Pellet was collected by centrifugation at max speed for 5 min at room temperature, isopropanol was carefully removed, and pellet was washed with 500 μL of 70 % (v/v) ethanol before centrifuging again at max speed for 5 min at room temperature;
9. 70 % (v/v) ethanol was removed and pellet resuspended in 100 μL of TE buffer (10 mM Tris, 1 mM EDTA, at pH 8);
10. To remove RNA from nucleic acid suspension, 1 μL of RNase (10 $\mu\text{g}/\mu\text{L}$) was added to suspension and sample was incubated for 20 min at 37 $^{\circ}\text{C}$;
11. 50 μL of ammonium acetate (7.5 M) was added and sample centrifuged at 13 000 rpm for 15 min at room temperature;
12. Supernatant was collected from each sample (150 μL) and 100 μL of isopropanol at 0 $^{\circ}\text{C}$ added and mixed gently;
13. Samples were centrifuged at 13 000 rpm for 15 min;
14. Pellet was collected by removing isopropanol and washed with 500 μL of 70 % (v/v) ethanol and re-centrifuged at 13 000 rpm for 15 min;
15. After air drying, pellet containing genomic DNA was resuspended in 50 μL of dH_2O ;
16. All samples were placed over-night at 4 $^{\circ}\text{C}$ before analyzing integrity and concentration and storing at -20 $^{\circ}\text{C}$.

S 2 – Silver staining protocol adapted from Bassam *et al.* 1991.

1. Glass containing polyacrylamide gel was placed in a recipient with 2 L of 10 % (v/v) acetic acid in dH₂O for 1 hour;
2. After discarding acetic acid solution, gel was washed three times with 2 L of dH₂O for 2 min each time;
3. 2 L of silver nitrate solution (2 g silver nitrate, 3 mL of 37 % (v/v) formaldehyde) was added and gel incubated for 30 min in the dark;
4. Silver nitrate solution was discarded the gel washed with 2 L of dH₂O for 10 s;
5. For visualization, a 2 L solution containing 60 g sodium carbonate, 3 mL of 37 % (v/v) formaldehyde and 1 mL sodium thiosulfate was added and gel gently shaken until bands were revealed;
6. Reaction was stopped by adding 1 L of 10 % (v/v) acetic acid to the recipient with the previous solution for at least 5 min;
7. Finally, the 3 L solution was discarded, the polyacrylamide gel washed in 2 L of dH₂O for 10 min and allowed to dry overnight at room temperature.

S 3 – RNA extraction protocol adapted from Chang *et al.* 1993.

1. Approximately 1 g of vegetative material in liquid nitrogen was ground to a fine powder with a mortar and pestle;
2. Samples were transferred to tubes containing 20 mL of pre-heated (at 65 °C) extraction buffer (2 % (w/v) CTAB, 2 % (w/v) PVP, 100 mM Tris-HCl pH 8, 25 mM EDTA) and 400 µL of 2-mercaptoethanol and well mixed.
3. 20 mL of chloroform /isoamyl alcohol (24:1) were added to each tube and mixed well until emulsion was formed;
4. Samples were centrifuged for 15 min at 4 000 rpm at 20 °C;
5. Supernatant was collected to new tubes and steps 3 and 4 were repeated;
6. 1/4 of the volume of LiCl (10 M) was added to the collected supernatant, mixed well and incubated over-night at on ice;
7. After incubation samples were centrifuged for 15 min at 4 000 rpm, at 4 °C;
8. Supernatant was discarded and the pellet was allowed to dry;
9. 1.5 mL of pre-heated (at 37 °C) SSTE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 1 M NaCl, 0.5 % (w/v) SDS) was added to the pellet and each sample was divided in two tubes;
10. 750 µL of chloroform / isoamyl alcohol (24:1) was added, mixed well and centrifuged for 10 min at 13 000 rpm;
11. Supernatant was removed and step 10 was repeated;
12. 2.5 volume of 100 % ethanol was added and well mixed;
13. Samples were incubated for 30 min at -80 °C, and then centrifuged for 30 min at 13 000 rpm, at 4 °C;
14. Supernatant was discarded and 250 µL of 70 % (v/v) ethanol was added;
15. Samples were centrifuged for 20 min at 13 000 rpm, at 4 °C;
16. Supernatant was discarded and the pellet was allowed to dry;
17. Finally 30 µL of dH₂O was added each sample and put in ice for 15 min, samples were stored at -80 °C.

