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Characterization and expression of cytokinin signalling genes in sulfur deficient grapevine (*Vitis vinifera* L.)

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Abstract

Sulfur (S) is an essential macronutrient for plant growth and development. *In vitro* grapevine *callus*, cells and shoots in culture media in the absence of sulfur (-S) respond markedly with a reduction of growth and shoot multiplication. This may result from an interference of -S with cytokinin signal pathway (CSP) or at shoot apical meristem (SAM) or axillary meristem (AM) identity level. Cytokinins are essential plant hormones that control various processes in plants. As in *Arabidopsis*, *Vitis* CSP is composed by receptors (HKs), phosphotransmitters (HPTs) and two types of response regulators (A-type and B-type RRs). Cells in -S in the presence of cytokinin show a downregulation of most CSP genes while -S without cytokinin leads to an upregulation of A-type RRs. CSP is not significantly affected by –S in *in* vitro shoots, so the multiplication inhibition can be caused by a downregulation of the expression of SAM and AM identity genes, respectively *STM* and *LAS*. *In vitro* conditions more similar to autotrophy as Temporary Immersion System, the scarce multiplication impairment must result from the reduction of B-type RRs transcription. As a whole the present work provides new insights on the crosstalk between –S and cytokinin signaling in *in vitro* grapevine model systems.

Keywords: Apical meristem, axillary meristem, cytokinin signalling pathway, *Vitis*, sulfur.

Resumo

O enxofre é um macronutriente essencial ao crescimento das plantas. *Callus*, células e "plantinhas" de videiras *in vitro,* quando colocadas na ausência de enxofre (-S), registaram uma redução da sua taxa de crescimento. Este fenómeno pode dever-se à interferência do –S na via de sinalização das citocininas (VSC) ou devido a uma alteração nos genes responsáveis pela identidade do meristema apical e axilar. A citocinina é uma hormona que controla vários fenómenos nas plantas. Tal como em *Arabidopsis*, a VSC em videira é constituída por receptores HKs, HPTs e dois tipos de RR (RR Tipo A e B). Células em –S e na presença de citocinina mostraram uma diminuição na transcrição dos gene da VSC, quando em meio sem citocinina os RR tipo A aumentam a sua expressão. Os genes VSC de "plantinhas" *in vitro*, não são significativamente afectados em –S, a inibição do crescimento pode dever-se à diminuição da transcrição dos genes de identidade do meristema apical e axilar, STM e LAS respectivamente. Em condições mais semelhantes ao autotrofismo, como em TIS, a redução no crescimento das plantas pode dever-se a uma diminuição da taxa de transcrição do RR tipo B. Este trabalho fornece novas perspectivas da relação entre o –S e a sinalização das citocininas em videira.

Palavras Chave: Enxofre, meristema apical, meristema axilar, via de sinalização das citocininas, videira.

Resumo alargado

O enxofre desempenha um papel fundamental nas plantas devido ao seu papel crucial no metabolismo. As plantas têm a capacidade de reduzir o SO_4^{2-} a S^{2-} que depois é assimilado sobre a forma de cisteína. A absorção a partir do solo é realizada por transporte activo usando dois tipos de transportadores, de alta ou baixa afinidade, sendo os transportadores de alta afinidade fortemente transcritos numa situação de carência de enxofre. A assimilação de enxofre inicia-se com a activação do sulfato, que é convertido em *APS*. Em seguida o *APS* é reduzido, numa reacção de dois passos, dando origem a S^{2-}. Por fim a cisteína incorpora o S^{2-} através da acção das enzimas *SAT* e *OASTL*. Recentemente todos estes genes foram também identificados em videira.

Videiras *in vitro,* num meio sem sulfato, apresentam uma redução no seu crescimento (branching). O peso fresco de *callus* sujeito à carência de sulfato sofreu uma redução acentuada em relação a cultura com sulfato. Durante as duas primeiras semanas a diferença entre as duas culturas não é significativa, só após a quarta semana as diferenças são significativas, com a diminuição do peso fresco na cultura sem sulfato. A taxa relativa de crescimento apresenta a mesma tendência. Os rebentos em caixa e em TIS apresentam a mesma tendência de diminuição do peso fresco e número de novos lançamentos, apesar do efeito de branching em TIS não ser tão acentuado. Este fenómeno pode dever-se a uma alteração da via do sinal das citocininas ou à alteração da expressão dos genes ligados à identidade do meristema.

A citocinina é uma importante hormona das plantas que controla vários aspectos do seu desenvolvimento como a divisão celular. A via do sinal das citocininas foi descoberta em várias espécies como *Arabidopsis*, milho e arroz. Em *Arabidopsis* o sinal desta hormona é transmitido pelo que é conhecido como “two-component system”. A hormona é percepcionado por receptores de membrana conhecidos como Histidina Cinase (HK), estes vão transferir um grupo fosfato ao componente seguinte da cadeia os Fosfotransmissores de Histidina (HPts), estes vão transferir novamente o grupo fosfato ao componente seguinte na via, os Reguladores de Resposta (RR) tipo-B, por sua vez estes actuam como factores de transcrição dos RR tipo-A que produzem uma determinada resposta à presença da citocinina. Em *Arabidopsis* existem 3 HKs, 5 HPts, 11 RRs tipo-B e 12 RR tipo-A. Com a descoberta do genoma da videira foi possível identificar os componentes da via de sinalização das citocininas. Na videira foram identificados 3 HKs (*VvCyt1*, *VvCyt2* e *VvCyt3*), quatro HPts (*VvHP1*, *VvHP2*, *VvHP3* e *VvHP4*), 4 RRs tipo-A (*VvRRa1*, *VvRRa2*, *VvRRa3* e

VvRRa4) e 6 RRs tipo-B (*VvRRb1*, *VvRRb2*, *VvRRb3, VvRRb4, VvRRb5* e *VvRRb6*). Através da análise filogenética podemos concluir que estes genes apresentam uma grande semelhança com os genes já descritos em outras espécies, tanto monocotiledónias como dicotiledónias, o que nos permite concluir que esta via foi conservada ao longo do período evolutivo das plantas.

A técnica de PCR em tempo real e largamente usada para estudar a expressão de genes em diferentes condições experimentais. Por isso escolhemos esta técnica para estudar o efeito da ausência de sulfato na via da sinalização da citocinina e nos genes ligados à identidade do meristema apical e axilar. Ao nível do receptor apenas os genes *VvCyt1* e *VvCyt2* responderam, na ausência de sulfato *VvCyt2* apresentou uma diminuição na quantidade de transcrito. Na ausência de sulfato e citocinina, apenas *VvCyt1* respondeu, também com uma redução da sua taxa de transcrição. No entanto a taxa de transcrição destes genes não foi alterada em plantas em meio gelificado (GM). Em células, os dois genes HPs (*VvHP2* e *VvHP3*) foram afectados pela carência de enxofre, diminuindo a sua taxa de transcrição. Nas células sem enxofre e sem citocinina o nível transcrição também diminuiu, indicando que o efeito combinado é semelhante ao de ausência de enxofre. Em GM os HPs não foram afectados pela diminuição do sulfato. Em células em cultura a expressão dos genes *VvRRa3* e *VvRRa4* foi também afectada pela carência de sulfato, mas a sua expressão foi afectada diferencialmente dependendo da presença ou ausência de citocinina no meio. Na presença de citocinina a expressão de *VvRRa4* diminuiu enquanto na ausência de citocinina o gene *VvRRa3* aumentou a sua expressão. Este resultado aparentemente contraditório pode ser explicado pela capacidade dos RR funcionarem como reguladores positivos ou negativos do sinal da citocinina. Em células e em GM os RRs tipo B não foram afectados pela ausência de enxofre ou de citocinina no meio; apenas em plantas em Sistema de Imersão Temporária (TIS) estes foram afectados, o que indica que a condição mais próxima da autotrófia afecta a sua taxa de transcrição levando a uma diminuição do sinal das citocininas.

A ausência de enxofre do meio pode também afectar a proliferação de células ao nível do meristema apical e axilar. Em videira foram identificados 3 genes ligados a identidade do meristema apical (*VvWus*, *VvCLV* e *VvSTM*). Apenas *VvSTM* e afectado pela ausência de sulfato, sofrendo uma redução da taxa de transcrição. Em *Arabidopsis* o *STM* é responsável pela manutenção de um nicho de células indiferenciadas no meristema apical. Com a redução da transcrição a planta perde a capacidade de repor estas células, não ocorrendo a renovação do meristema apical. Ao nível do meristema axilar foram identificados 5 genes (*VvBRC1*, *VvBRC2*, *VvLAS*, *VvRAX* e *VvREV*). Em plantas em GM apenas *VvBRC1*,

VvBRC2 e *VvLAS* foram afectados pela diminuição de enxofre no meio, sofrendo uma redução da sua taxa de transcrição. Em *Arabidopsis* os genes *BRC1* e *BRC2* são responsáveis pela diminuição do número de lançamentos laterais logo se a sua taxa de transcrição diminui ocorre um aumento do número de lançamentos laterais. Em *Arabidopsis,* o gene *LAS* é necessário para a iniciação do meristema. Os resultados obtidos confirmaram que a diminuição da transcrição do gene *VvLAS* conduziu à incapacidade da planta de obter um meristema funcional, apesar da diminuição na transcrição dos genes *VvBRC1* e *VvBRC2*, o que indica que estes genes actuam posteriormente ao *VvLAS*.

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Abbreviations List

Act	Actine
AM	Axillary meristem
APS	Adenosine 5'-phosphosulfate
BA	6-benzylaminopurine
BRC	*BRANCHED*
cDNA	complementary DNA
CHASE	Cyclases/histidine kinases associated sensory extracellular
Chla	Chlorophyll a
Chlb	Chlorophyll b
CLV	*CLAVATA*
C_T	Threshold cycle
CTAB	Hexadecyltrimethylammonium bromide
CZ	Central zone
DMSO	Dimetyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
GARP	DNA-binding motif
GM	Gel medium
GSH	Reduced Glutathione
HAST	High-affinity sulfur transport

HK	Histidine Kinase recepter
HPt	Histidine phosphotranfer protein
IPT7	*ISOPENTENYL TRANAFERASE* 7
K_m	Michaelis-Menten constant
KNOX1	KONTTED1-like homeobox
LAS	*LATERAL SUPRESSOR*
LAST	Low- affinity sulfur transport
L1	Epidermal layer
L2	Subepidermal layer
L3	Corpus
MS	Murashige and Skoog medium
NAA	α-naphthaleneacetic acid
OAS	*O*-acetyl serine
OASTL	*O*-acetyl-L-serine(thiol)-liase
PVP-40T	Polyvinylpyrrolidone
PZ	Peripheral zone
qrt RT-PCR	Quantitative real-time PCR
RAX	*REGULATOR OF AXILLARY MERISTEMS*
REV	*REVOLUTA/INTERFASCICULAR FIBERLESS*
RGR	Relative Growth Rate
RT-PCR	reverse transcriptase polymerase chain reaction
RR	Response regulator protein
RZ	Rib zone

S	Sulfur
SAM	Shoot apical meristem
SAT	Serine acetyl transferase
STM	*SHOOT MERISTEMLESS*
TAE	Tris-acetate EDTA buffer
TIS	Temporary immersion system
Tot Chl	Total chlorophyll
VvBRC	*Vitis vinifera* BRANCHED
VvClv	*Vitis vinifera CLAVATA*
VvCyt	*Vitis vinifera* cytokinin receptor
VvHP	*Vitis vinifera* histidine phosphotransfer
VvLAS	*Vitis vinifera LATERAL SUPRESSOR*
VvRAX	*Vitis vinifera REGULATOR OF AXILLARY MERISTEMS*
VvREV	*Vitis vinifera REVOLUTA/INTERFASCICULAR FIBERLESS*
VvRR	*Vitis vinifera* response regulators
VvSTM	*Vitis vinifera SHOOT MERISTEMLESS*
VvWUS	*Vitis vinifera WUSCHEL*
WUS	*WUSCHEL*
2,4-D	2,4-dichlorophenoxy-acetic acid

1. Introduction

1.1. Sulfur

Sulfur (S) is the 14th more abundant element on earth crust (Chalson *et al.*, 1992), the 9th and least abundant essential macronutrient in plants (Saito, 2004). The inter-conversion of oxidized and reduced sulfur states, the biogeochemical sulfur cycle, depends mainly on microorganisms (Falkowski *et al.*, 2008) and plants. The inorganic forms of S in soil consist mainly of sulfates (SO_4^{2-}) (Mengel and Kirkby, 1978). Assimilatory reduction of sulfate ion integrates, together with O_2 bioproduction, CO_2 fixation, nitrate ion reduction and N_2 fixation, the biological processes essential to aerobic life. In the reduced state, S is the key element of the amino acids cysteine and methionine (Xavier and LeGall, 2007). It is commonly accepted that the key function of sulfur is to provide disulfide bonds between amino acids within proteins. The thiol group of cysteine radical is fundamental for protein structure and function. Other thiol compounds more stable than cysteine, e.g. the tripeptide glutathione (GSH), concur to the cell redox regulation (Rouhier *et al,.* 2008). Plants, yeasts and some bacteria can reduce sulfur from the oxidation/reduction state of SO_4^{2-} (+6) to the sulfide (S^{2-}) state (-2).

Plants are able reduce SO_4^{2-} to S^{2-} and incorporate it into cysteine; then the greater part of sulfate taken up by plants is used for protein synthesis (Brunold, 1976). This explains the involvement of sulfur in most essential metabolic pathways and its key role in plant growth.

1.1.1. Sulfur nutrition and plant defense against pathogens

Plants do not accumulate or remobilize S-reserves (Mengel and Kirkby, 1978). In the past, the sulfur used by crop plants resulted from two sources: sulfur-containing fertilizers and/or sulfur in rainfall (Jolivet, 1993). Due to environmental policies both these sources were significantly reduced in the last 25 years: atmospheric sulfur deposition significantly decreased and many of the currently used mineral fertilizers lack sulfur (Blake-Kalff *et al.*, 2000). Recent studies indicate that sulfur deficiency can be a limiting factor to crop yield and

quality (Saito, 2004; Hawkesford, 2005). Therefore, former research on plant adaptation to excessive inputs of sulfur due to aerial pollution moved now into the effects of S-deficiencies.

It is demonstrated that sulfur nutrition exerts a positive influence of sulfur nutrition on plant health (Bloem *et al.,* 2007). Elemental sulfur (S^0) is probably the oldest pesticide, with references as old as 1000 BC (Williams and Cooper, 2004). Unexpectedly for eukaryotes, it was unraveled that some plant species produce S^0 as a component of the defense system against vascular pathogens (Williams *et al.,* 2002).

In the group of defense compounds are included several S-secondary plant metabolites (Hell and Kruse, 2007), namely glucosinolates and alliins (Schnug, 1997).

1.1.2. Sulfur in grapevine nutrition and health

References to sulfur use are found since ancient times, as in the Bible and in Greek and Roman literature. As early as more than 2000 years ago, Romans discovered the beneficial effects of sulfur as a potent agent against plant pathogens and refer the application of elemental sulfur in the vineyards (Rausch, 2007).

The effect of sulfur on plant growth, productivity and product quality mostly relates sulfur nutrition in interaction with nitrogen (Brunold, 1976; Byers *et al.*, 1987; Schnug, 1997). In grapevine xylem sap while nitrate is the major anion sulfate, chloride and phosphate increase after N fertilizer treatments (Peuke, 2000). The protective effect of elemental S against grapevine pests and diseases has been mostly reported after foliar application although S-fertilization can substitute for fungicide application in crop protection from pest attack (Bloem *et al.*, 2007).

1.2. Sulfate as the main sulfur source

1.2.1. Grapevine sulfate uptake and sulfate transporters

Sulfate is acquired by plant roots from the soil by a multiphase rate mechanism (Clarkson *et al.* 1993). Two kinds of transporters mediate the initial uptake and the distribution of sulfate throughout the plant: one with low K_m (10 μM) assuring a high-affinity sulfate transport (HAST), and another with much higher K_m responsible for the low-affinity sulfate transport (LAST) (Amâncio *et al.*, 2009).

The primary response of numerous plant systems under sulfur depletion is a clear upregulation (or derepression) of HAST, at the transcription level (Smith *et al.*, 1995 1997, Leustek *et al.*, 2000; Takahashi *et al.*, 1997 2000; Shibagaki *et al.*, 2002; Yoshimoto *et al.,* 2002). The raise in the expression of sulfate transporter protein leads to an increase in uptake capacity (Hawkesford, 2000). Conversely, sulfate repletion leads to the down-regulation (or repression) of the transporters transcription (Maruyama-Nakashita *et al.*, 2004). Apparently, the regulation imposed by sulfur-status at the molecular level is highly coordinated with the physiological responses, either in cells (Hatzfeld *et al.*, 1998; Clarkson *et al.*, 1999) or at whole plant level (Clarkson *et al.*, 1993; Smith *et al.*, 1997).

The sulfate transporter sequences from different plant species, available in public databases, were organized into 5 groups based on the predicted protein sequences (Hawkesford, 2003). In Group 1 includes genes for HAST regulated by S external conditions. In *Arabidopsis thaliana* genome, three different sulfate transporters were identified (Yoshimoto *et al.*, 2003). Previously to *Vitis* genome release, a homologous sequence from *V. vinifera* cv Touriga Nacional *VvST* (EF155630) was obtained by RT-PCR using degenerated primers (Tavares *et al.*, 2008). Through its protein (and nucleotide) sequence but also the molecular and physiological data, *VvST* was assigned to sulfate transporter Group 1.

The grapevine genome release (Jaillon *et al.*, 2007, Velasco *et al.*, 2007) made it possible to identify nine protein sequences related to the sulfate transporter family. Phylogenetic analysis showed that these sequences can be assorted to three of the five sulfate transporter family groups (Amâncio *et al.*, 2009).

The common characteristic among Group 2 sulfate transporters is its low affinity to sulfate, expressed mainly in vascular tissue of both root and shoots tissues. *V. vinifera* only has one isoform assigned to Group 2 (*VvST2*), that is expressed both in roots and isolated cells (Tavares *et al.*, 2008).

Sulfate uptake by *V. vinifera* cells was significantly affected (Amâncio *et al.*, 2009) under a time scale similar to that described previously for maize cells (Clarkson *et al.*, 1999). In other systems like roots, after 15 days without S, the influx was twice the value of +S plants. The abundance of *VvST* mRNA matched the derepression of sulfate uptake capacity, suggesting a transcriptional regulation of the sulfate transport in response to S availability (Maruyama-Nakashita *et al.*, 2004).

A large and diverse number of sulfate transporters isoforms has been assigned to Group 3 (Hawkesford and De Kok 2006). *In Arabidopsis* only one isoform is characterized to date, which, apparently, is not a functional transporter but can contribute to sulfate uptake when co-expressed with a Group 2 sulfate transporter (Kataoka *et al.*, 2004). In *V. vinifera* genome six sequences fall to this group, confirming an apparent redundancy. Five out the six Group 3 *V. vinifera* sulfate transporters were expressed in roots and in culture cells, under a pattern equivalent to other Group 3 sulfate transporters identified in other species (Amâncio *et al*., 2009).

1.3. Sulfate assimilation

The assimilation of sulfate is, with carbon fixation and nitrogen assimilation, one of the basic pathways used for the incorporation of inorganic elements into the organic molecules that drive cell metabolism. Up to 70% of the total sulfur content of plants is in the form of cysteine and methionine and this one's mainly incorporated into proteins (Hankesford and De Kok, 2006). Plant SO_4^{2-} assimilation involves three main steps: sulfate activation, sulfate reduction and sulfide assimilation and cysteine synthesis.

Sulfate (SO_4^{2-}) is the most oxidative and thus stable form of sulfur present in the soil. Prior to reduction, sulfate is subjected to activation to adenosine 5'-phosphosulfate (5'-adenylylsulfate [APS]) for further conversion. Since the reaction equilibrium of ATP-S favours

the reverse reaction (ATP and SO_4^{2-} formation) the activity of ATP-S depends on the consumption of APS in further reactions (Saito 2004).

The second step in sulfur assimilation is sulfate reduction. In plants, recent lines of evidence demonstrate that the two step-reduction of SO_4^{2-} (S: +6) to S^{2-} (S:-2) is carried out by GSH-APS reductase (two electrons) (GSH:APS sulforeductase,) and the plastid enzyme sulfite reductase (S^{2-}: ferredoxin oxidoredutase, sulfite reductase, SIR) (Gutierrez-Marcos *et al.*, 1996, 1997; Bick and Leustek 1998). The first reduction step is equivalent to the bacterial formation of free sulfite (SO_3^{2-}) as a free intermediate. The reduction is completed by the production of sulphide S^{2-} by the transfer of 6 electrons from reduced ferredoxin to sulfite to form sulphide (Amâncio *et al.*, 2009).

Cysteine incorporates S^{2-} by the activity of the complex serine acetyl transferase (SAT) and *O*-acetyl-serine sulfydrilase (*O*-acetyl–L-serine(thiol)-liase, *O*ASTL). The level of free cysteine in plants is very low but the flux can be quite high (Höfgen and Hess 2007). The only direct carbon/nitrogen precursor for cysteine is *O*-acetyl serine (*O*AS), which results from the acetylation of serine by SAT. This substrate incorporates S_2^- into cysteine in a reaction catalysed by *O*ASTL (Droux *et al.*, 1998).

1.3.1. Regulation of sulfate assimilation by sulfur availability

Removal of S supply cause an increase in the protein activity or mRNA pools of some enzymes responsible for the uptake and the assimilatory pathway, such increase is observed after several days in whole plants (Hell *et al.*, 1997; Buchner *et al.*, 2004) or several hours in cell suspensions (Hatzfeld *et al.*, 1998). Following re-supply of SO_4^{2-} all fall in parallel with cysteine and GSH increase. In most plant systems analyzed so far, sulfate, cysteine, and GSH are described as negative regulators and OAS as positive regulator of sulfur genome (Droux, 2004). GSH-APS reductase is thought to be a prime regulation point of the pathway (Vauclare *et al.*, 2002), since its activity and RNA increase concomitantly with S starvation and with stresses that increase the demand for GSH and then for cysteine (Amâncio *et al.*, 2009).

1.3.2. Grapevine genes for sulfate assimilation enzymes

Partial sequences of *V. vinifera* genes encoding for APR, SAT and OASTL were identified. They were cloned and deposited at GenBank, respectively (EU275236), (EU275238) and (EU275237) (Amâncio *et al.*, 2009). Subsequently to grapevine genome sequencing (Jaillon *et al.*, 2007; Velasco *et al.*, 2007), sequences of genes for putative isoforms of sulfate assimilation enzymes were identified in databases.

For APS two isoforms were identified in *V. Vinifera* genome, *VvATPS-1* and *VvATPS-2*. Unlike *A. thaliana* where 4 different *APSR* isoforms were described, only one isoform is present in grapevine, *VvAPSR*. As in *Arabidopsis* one sole isoform of sulfite reductase is present in *Vitis*, *VvSIR* (Amâncio *et al.*, 2009).

1.3.3. Expression of V. vinifera sulfur assimilation genes

The analysis of the expression of genes for sulfate metabolism enzymes in response to sulfate depletion in grapevine isolated cells, but also in roots and leaves, showed that the relative abundance of *VvATP-S1, VvSr* and particularly *VvAPR,* are up-regulated in the three systems (Amâncio *et al.*, 2009), confirming the crucial role of APR in sulfur metabolism pathway (Vauclare *et al.*, 2002).

1.4. Cytokinin

Cytokinins are essential plant hormones that control various aspects of plant growth and development such as cell division, shoot formation, senescence and chloroplast development (Ito and Kurata, 2006). Cytokinins were discovered during the 1950s due to their ability to induce plant cell division (Miller *et al.*, 1955). The most abundant cytokinins are adenine derivatives substituted at the N^6-position with an isoprenoid side chain (Heyl *et al.*, 2006). Conclusions about the biological functions of cytokinins have mainly been derived from studies on the consequences of exogenous cytokinin application or endogenously

enhanced cytokinin levels (Klee and Lanahan, 1995). The previously widely accepted idea that cytokinin was synthesized only in root tips, is now overturned. This hormone has coordinated function as long-distance messenger as well as local signalling; cytokinin is synthesized and act at various sites in a plant body (Sakakibara, 2006). In the control of outgrowth and dormancy of axillary buds, the mutual regulation of auxin, ABA, and cytokinin has been proposed to play a central role (Shimizu-Sato and Mori, 2001). Although an antagonistic role of auxin and cytokinin in the regulation of axillary bud outgrowth has been postulated for a considerable time, little is known. Recent studies revealed that one role of apex-derived auxin in apical dominance is to repress cytokinin biosynthesis in the nodes and that after decapitation cytokinins are locally synthesized in the stem rather than being transported to the stem from the roots (Tanaka *et al.*, 2006).

Recent studies, carried out by Hwang and Sheen (2001), revealed genes implicated in the mechanism of cytokinin perception and signalling in *Arabidopsis* (Heyl and Scholuling, 2003). Signal transduction systems function as intracellular information-processing pathways that link external stimuli to specific adaptive responses (West and Stock, 2001). The signalling system, besides the membrane bound receptor kinase, which senses the signal and autophosphorylates, consists of phosphotransmitter protein and a response regulator, which upon phosphorylation, activates the transcription of its target genes or initiates another output reaction (West and Stock, 2001).

1.4.1. Signal perception and transduction

In bacteria, phosphorylation on a nitrogen atom of a histidine (His) residue and on an acyl group of an aspartate (Asp) residue is predominantly used to transmit the cytokinin signalling (Kakimoto, 2003; Klumpp and Krieglstein, 2002). The mode of signalling that uses this type of phosphorylation has been referred to as the “two-component system”. The signalling pathways, referred as “two-component system” are structured around two conserved proteins: a histidine protein kinase (HK) and a response regulator protein (RR) that are phosphorylated. Phosphotransfer from HK to RR results in the activation of the later and generation of the output response of the signalling pathway (West and Stock, 2001).

A more complex version of this two-component phosphotransfer scheme includes the His-Asp-His-Asp phosphorelay. This phosphorelay involves multiple phosphotranfer steps and often more than two proteins (Perraud *et al.*, 1999), as verified by *Arabidopsis* and grapevine (this research). In this system, in addition to HK and RR, also consists in a histidine phosphotransfer protein (HPt), which mediates the signal between the HK and RR (Suzuki *et al.*, 2000).

Three *Arabidopsis* HK genes (*AHK4/CRE1*, *AHK2* and *AHK3*) (Hwang and Sheen, 2001), three maize HK genes (*ZmHK1*, *ZmHK2* and *ZmHZ3a*) (Asakura *et al.*, 2003) and five HK in rice (*OHK1*, *OHK2*, *OHK3*, *OHK4* and *OHK5*) (Ito and Kurata, 2006) were described. The identification of orthologs for cytokinin signalling components in other plant species suggests evolutionary conservation of this pathway (Müller and Sheen, 2007). In *Arabidipsis*, the three cytokinin receptor genes differ in their expression location: *CRE1/AHK4* is mainly expressed in the roots whereas *AHK2* and *AHK3* are expressed in all major organs.

The analysis of *Arabidopsis* signalling pathway resulted in a model that distinguishes four major steps: (i) cytokinin sensing and initiation of signalling by receptor HKs; (ii) phosphoryl group transfer to HPts and their translocation to the nucleus; (iii) phosphotransfer to nuclear B-type RRs, which activate transcription; and (iv) negative feedback through cytokinin-inducible A-type RRs, which are the products of early cytokinin target genes (Fig. 1) (Müller and Sheen, 2007).

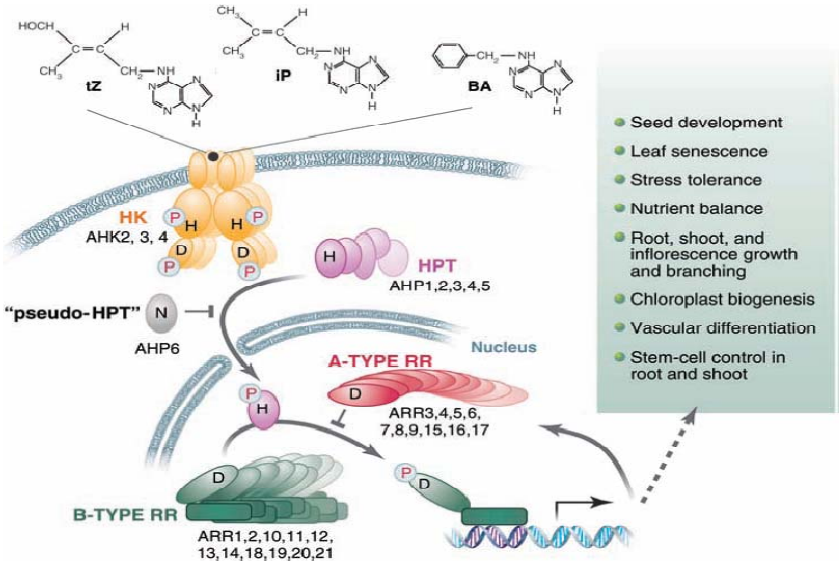

Fig. 1 Model for the cytokinin multistep two-component circuitry through histidine (H), and aspartate (D) phosphorelay, involving histidine-kinase receptors (HK), phosphotransfer proteins (HPT), a "pseudo–HPT" with an asparagine (N) instead of the D, and A-type and B-type RRs (Müller and Sheen, 2007).

The large majority of HKs are membrane-bound, homodimeric proteins with an amino-terminal periplasmic sensing domain that is coupled to a C-terminal cytoplasmic kinase domain (Fig. 2) (West and Stock, 2001). All HKs share a domain in the predicted extracytoplasmic region, designated CHASE, which is the putative recognition site for cytokinin (Ueguchi *et al.*, 2001; Du *et al.*, 2007).

The triple mutant for these three HK genes showed various cytokinin-related development defects and no cytokinin response, but is not lethal (Higuchi *et al.*, 2004). This suggests that either cytokinin is not essential to *Arabidopsis* growth or another unknown signalling pathway of cytokinin may exist and support the cytokinin-dependent growth of *Arabidopsis* (Ito and Kurata, 2006).

The HPts perceive a phosphate group from HKs and translocate the phosphate from cytoplasm to the nucleus where it is transferred to RRs. In *Arabidopsis*, AHPs are predominantly located in the cytoplasm and only a minor proportion in the nucleus (Tanaka *et al.*, 2004). Importantly, it has been shown that upon induction by cytokinin some AHPs (*AHP1* and *AHP2*) localize specifically and transiently to the nucleus, indicating it as cytoplasmic-nuclear shuttles, so HPts serves as a crucial intermediate in a His-to-Asp phosphorelay pathway by acquiring and transferring a phosphoryl group from and to a receiver (Suzuki *et al.*, 2000). Recent findings have added some twists to the pathway. Aside from its kinase function, a cytokinin receptor was found to exhibit phosphatase activity that removes phosphoryl groups from AHPs when no cytokinin is bound. Many prokaryote HKs associated with phosphorelay systems that need to be shut off quickly have phosphatase activity. In *Arabidopis*, the HK phosphatase activity may ensure that, in the absence of cytokinin, the pathway is quickly and completely inactivated (Mähönen *et al.*, 2006).

The RRs are classified in two groups: A-type RR and B-type RR. The B-type RRs have a phosphorelatable receiver domain at their N-terminus and a GARP DNA-binding domain in the midpoint of the sequence; when phosphorylated the activated B-type RRs induce or repress the expression of target genes (Hwang and Sheen, 2001; Sakai *et al.*, 2001). B-type ARRs were found in de nucleus and the structural analysis confirmed they bind to DNA. The predicted secondary structure of the binding domain, a helix-turn-helix motif similar to homeobox proteins, recognizes the major groove of DNA (Hosoda *et al.*, 2002). The DNA motif optimal for binding is 5′-(A/G)GAT(T/C)-3′ with the GAT motif, in the middle, is of special importance (Sakai *et al.*, 2000; Lohrmann *et al.*, 2001; Hosoda *et al.*, 2002). 5′-AGATT-3′ was found to be optimal for *ARR1*, *ARR2* and *ARR10* (Sakai *et al.*, 2000; Hosoda *et al.*, 2002), whereas *ARR11* binds preferentially to 5′-GGATT-3′ (Imamura *et al.*, 2003).

Unlike the expression pattern of A-type ARR genes, the steady-state levels of B-type ARR transcripts are apparently not affected by the application of cytokinin or other plant hormones (Fig. 2).

The A-type RRs have the receiver domain but lack the GARP domain (Fig. 2). Expression of A-type RRs is induced by cytokinin, and the induction is mediated by B-type RRs (D'Agostino *et al.*, 2000). A-type RRs display properties of cytokinin primary-response genes: the elevation of the steady-state level of transcript occurs within 10 min of exogenous cytokinin application, the rapid induction is specific for cytokinin (D'Agostino *et al.*, 2000).

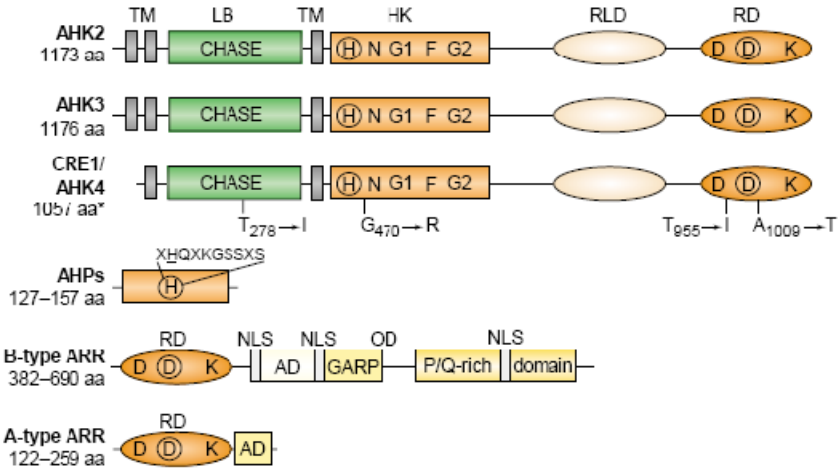

Fig. 2 Structures of cytokinin receptors and other proteins of the cytokinin signalling pathway. Amino acids that participate in the phosphorelay are circled. Other characteristic consensus motifs are also indicated. Abbreviations: aa, amino acids; AD, acidic domain; CHASE, cyclases/histidine kinases associated sensory extracellular; GARP, DNA-binding motif; HK, histidine kinase; LB, putative ligand binding domain; NLS, nuclear localisation signal; OD, output domain; RD, receiver domain; RLD, receiver-like domain; TM, transmembrane domain (Heyl and Schmülling, 2003).

1.4.2. *Cross-talk between mineral nutrition and cytokinin signalling*

As referred above, the primary response of most plant systems under sulfur deficiency is a clear upregulation (or derepression) of sulfate transporters at the transcription level (Smith *et al.*, 1995, 1997; Leustek *et al.*, 2000; Takahashi *et al.*, 1997, 2000; Shibagaki *et al.*, 2002; Yoshimoto *et al.*, 2002). Conversely, sulfate repletion leads to the down-regulation (or repression) of the transporters transcription (Maruyama-Nakashita *et al.*, 2004).

It was verified in *Arabidopsis* that cytokinin down-regulates the expression of a HAST gene and the sulfate uptake which are induced by sulfur limitation and repressed by the presence of a sulfur source. Maruyama-Nakashita *et al.* (2004), proposed that cytokinin could act as a negative regulator of sulfur acquisition, suggesting the existence of at least two independent modes of regulation for sulfate acquisition, one induced by sulfate depletion and one dependent on cytokinin.
More convincing evidence for the involvement of hormone signalling components in –S response came from the genetic study of the cytokinin receptor mutant *cre1* (Maruyama-Nakashita *et al.*, 2004). The potential role of cytokinin in –S response was suggested by a study in which the –S activated expression of the β-subunit of seed storage protein β-conglycinin show to be promoted by cytokinin (Ohkama *et al.*, 2002). On the other hand the mutation in *CRE1* reduced the cytokinin suppression of both the *SULTR 1;2* expression and S uptake. This demonstrates that cytokinin perception plays a negative role at least in regard to sulfate transport (Dan *et al.*, 2007).

However, when zeatin was applied to sulfur-sufficient *Arabidopsis* an accumulation of sulfate transporter and APS reductase transcripts was observed (Ohkama *et al.*, 2002), pointing to a positive regulation by exogenous cytokinin on the expression of sulfate-responsive genes.

If cytokinins do, in fact, are the sole mediators of sulfur starvation signal to regulate the expression of sulfur responsive genes, its concentration in –S tissues was expected to increase. However, in leaf tissues, cytokinin concentration did not increase significantly after two days of sulfate starvation suggesting that it is unlikely that, at least in leaves, cytokinins mediate directly sulfur-deficiency (Ohkama *et al.*, 2002).

Other macronutrients, *e.g.* phosphate (P), may respond to the application of cytokinin. It has been shown that exogenous application of cytokinin represses the induction of many P starvation-responsive genes in *Arabidopsis* (Martin *et al.*, 2000) and this effect is attenuated in *cre1* mutants, implicating cytokinin in the negative regulation of P starvation responses (Franco-Zorrilla *et al.*, 2002).

1.5. Shoot apical meristem (SAM)

Post-embryonic development in higher plants is characterized by the reiterative formation of lateral organs from the flanks of the apical meristems (Steeves and Sussex, 1989). The shoot apical meristem (SAM), initially formed during embryogenesis, is located at the shoot apex and leaves, stems and axillary meristems are produced from its derivative cells (Shani *et al.*, 2006). The SAM contains a population of pluripotent stem cells, with three primary functions: lateral organs, such as leaves, are produced from the peripheral regions of SAM; the basal region cells contribute to the formation of the shoot axis; and the stem cells of SAM must replenish those regions from which cells have been recruited and maintain the pool of stem cells required for further growth (Lenhard and Laux, 1999; Bowman and Eshed, 2000). The shoot apical meristem can be divided into different histological zones. This division is based in two different classifications, one based in histological analyses of shoot apical meristem (Lenhard and Laux, 1999) and the other based in the clonally distinct layers of cells in the zone of the shoot apical meristem (Satina *et al.*, 1940). Based in the first classification, three distinct zones of SAM are defined by cytoplasmatic densities and cell division rate: the peripheral zone (PZ), the central zone (CZ) and the rib zone (RZ) (Shani *et al.*, 2006). Lateral organs are produced from cells recruited from PZ. The CZ, at the summit of the SAM, contains self-maintaining, slowly dividing cells, which provides initials for the PZ (Shani *et al.*, 2006). The shoot axis tissue is derived from cells recruited from the RZ. The CZ acts as a reservoir of stem cells, which replenish both the peripheral and rib zone, as well as maintaining the integrity of the central zone (Fig. 3 a) (Bowman and Eshed, 2000).

The SAM is also composed of clonally distinct layers of cells (Satina *et al.*, 1940). The fact that the peripheral and central zone, as well as the lateral organs produced, contain cells from the three clonally distinct layers indicates that communication between cell layers is required to coordinate developmental processes. For example, leaves in most eudicot species are composed of derivatives from the epidermal layer (L1), the subepidermal layer (L2) and corpus (L3) (Fig. 3 b) (Satina *et al.*, 1941; Bowman and Eshed, 2000). One of the earliest markers of leaf initiation from the PZ is the periclinal cell divisions in specific regions in L2. Cells in L1 and L3 adjust their growth accordingly, with the entire region acting coordinately to produce a leaf primordium (Bowman and Eshed, 2000).

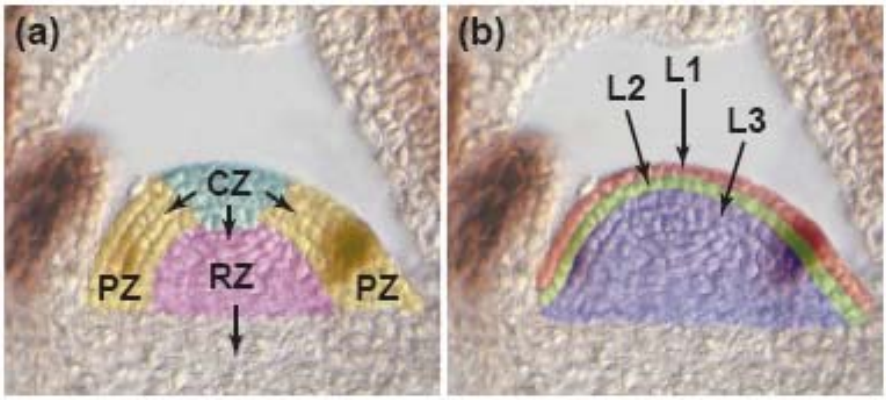

Fig 3 Histology of the shoot apical meristem (SAM). a) zones of SAM defined by cytoplasmic densities and cell division rate: peripheral zone (PZ); central zone (CZ) and rib zone (RZ); b) SAM defined by different clonally distinct layers of cells: epidermal layer (L1); subepidermal layer (L2) and Corpus (L3) (Bowman and Eshed, 2000).

Since 1957, after the work of Skoog and Miller, that the role of phytohormones in controlling the SAM differentiation is suggested. However, the pleiotropic nature of hormone action has made it difficult to understand exactly how hormones act to pattern development (Hay *et al.*, 2004). To this contributes the different effect of hormones depending on their concentration (Skoog and Miller, 1957) and may have opposite effects on the same process in different species (Grant *et al.*, 1994). However, hormones such as auxin and cytokinin appear to display dynamic concentration gradients in SAM and in other plant tissues (Shani *et al.*, 2006). Recent molecular and imaging studies unraveled information on the endogenous spatial and temporal distribution of plant hormones.

1.5.1. Regulation of shoot apical meristem

Plant development depends on the continuous activity of meristem to produce organs throughout plants life. In the past decade, the genetic dissection of plant hormone biosynthesis and signalling pathways has offered new opportunities for studying the role of hormones (Hay *et al.*, 2004). Several groups of transcription factors have been shown to take part in SAM differentiation. KNOTTED1-like homeobox (*KNOX1*) proteins are expressed in specific patterns in the SAM of different plant species (Shani *et al.*, 2006). The control of hormone biosynthesis by the KNOX class of transcription factors has recently emerged as an example of perhaps unexpected interactions between hormones and developmental genes (Sakamoto *et al.*, 2001; Hay *et al.*, 2002; Hay *et al.*, 2004).

KNOTTED1 defines the first homeobox gene family to be isolated in plants (Vollbrecht *et al.*, 1991). The *Arabidopsis* class 1 *KNOX* gene subfamily comprises *SHOOT MERISTEMLESS (STM), KNAT1/BREVIPEDICELLUS (BP), KNAT2* and *KNAT6* (Lincoln *et al.*, 1994; Dockx *et al.*, 1995; Long *et al.*, 1996; Semiarti *et al.*, 2001; Dean *et al.*, 2004; Scofield *et al.*, 2007). The gene *STM* is required for the initiation and maintenance of the shoot apical meristem in *Arabidopsis.* One of the earliest indicators of a switch in fate from indeterminate meristem to determinate leaf primordium is the down-regulation of *KNOX1* genes orthologous to *STM* in the incipient primordia (Uchida *et al.*, 2007). Embryos homozygous for strong loss-of-function mutations in the *STM* form cotyledons and other embryonic structures but fail to establish a population of self-renewing stem cells (Long *et al.*, 1996). Another early gene expressed is *WUSCHEL* (*WUS*), whose expression, in mature shoot apical meristem, is limited to a small group of cells underneath the outer three layers (L3). The *WUS* expression pattern gradually becomes limited to deeper regions of the shoot apical meristem, since it is required to produce the stem cell maintenance signal (Mar Castellano and Sablowski, 2005).

WUS expression is under negative control by the *CLAVATA* genes (*CLV1*, *CLV2* and *CLV3*), which encode components of a presumed receptor-kinase signal transduction pathway (Fletcher *et al.*, 1999; Lenhard *et al.*, 2002). Stem cells express *CLV3*, and signalling of *CLV3* through the *CLV1/CLV2* receptor complex restricts *WUS* activity. Homeostasis of the stem cell population may be achieved through feedback regulation, whereby changes in stem cell number result in corresponding changes in *CLV3* expression levels, and adjustment of *WUS* expression via the *CLV* signal transduction pathway. It was found that the expression of *CLV3* depends only of *WUS* function in the embryonic shoot meristem. At later developmental stages, *WUS* promotes the level of *CLV3* expression, together with *STM*. Within a meristem, competence to respond to *WUS* activity by expressing *CLV3* is restricted to the meristem apex (Brand *et al.*, 2000). In *clv* mutants, the SAM enlarges progressively by the accumulation of stem cells (Fletcher *et al.*, 1999; Lenhard *et al.*, 2002) and this enlargement appears to be a consequence of ectopic *WUS* expression in more apical and lateral cells in *clv* mutant SAMs (Schoof *et al.*, 2000). This has led to a model in which stem cell maintenance is regulated by a negative feedback loop mediated by the *WUS* and *CLV3* genes, with the organizing centre signalling to the apical neighbours to specify them as stem cells, which in turn signal back to restrict the size of the organizing centre (Brand *et al.*, 2000; Schoof *et al.*, 2000; Lenhard *et al.*, 2002). In summary, the earliest acting genes (*WUS* and *STM*), are required for the establishment or maintenance of stem cell fate or alternatively, the repression of differentiation, whereas later expressed genes might be

involved in regulating the size of the central zone (*CLV1*) (Bowman and Eshed, 2000) (Fig. 4).

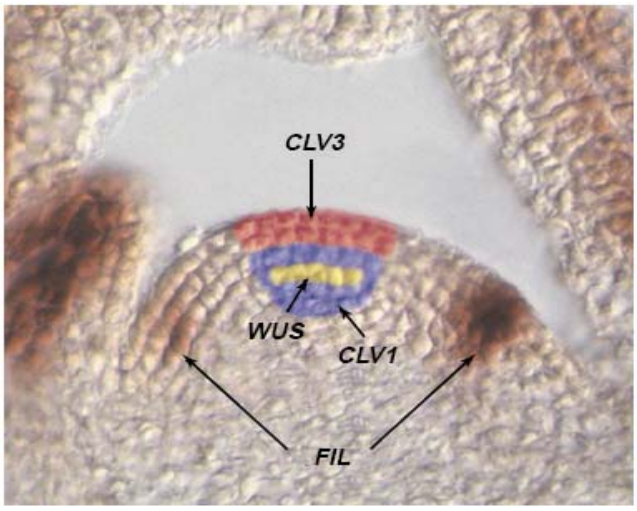

Fig. 4 Expression patterns of genes involved in maintaining the integrity of the central zone. CLAVATA3 (CLV3), CLAVATA1 (CLV1) WUSCHEL (WUS) and FILAMENTOUS FLOWER (FIL) (Bowman and Eshed, 2000).

1.5.2 Hormones and shoot apical meristem

Recent studies have revealed the relationships between genes for transcription factors and a variety of hormones like cytokinin, gibberellins and auxin in the shoot apical meristem (Shani *et al.*, 2006).

Cytokinin positively regulates cell division (Skoog and Miller, 1957; Riou-Khamlichi *et al.*, 1999). Recent advances in understanding the cytokinin-signal cascade in *Arabidopsis* enabled an assessment of the role of cytokinin in SAM function. This analysis focused on the triple mutant for the genes encoding cytokinin receptors (*AHK2*, *AHK3* and *AHK4/CRE1*) (Riefler *et al.*, 2006). This mutant displayed pleitropic phenotypes including a dramatic reduction in meristem size (Shani *et al.*, 2006).

The involvement of KNOX1 proteins and cytokinin in the response of SAM has been suggested by Hay *et al.* (2004). Two studies provided direct molecular evidence for the positive regulation of cytokinin biosynthesis by KNOX1 proteins in *Arabidopsis*. Ectopic activation of several KNOX1 proteins, including STM, in transgenic *Arabidopsis* plants result in a rapid increase in the expression of the cytokinin biosynthesis gene *ISOPENTENYL TRANSFERASE 7* (*IPT7*) (Jasinski *et al.*, 2005; Yanai *et al.*, 2005).

Arabidopsis type-A response regulators (RRs) are cytokinin-induced negative regulators of cytokinin signal (Ferreira and Kieber, 2005). Overexpression of a constitutively active form of *ARR7* caused variable phenotypic aberrations, the most severe of which was the early meristem determination, similar to that of *wus* mutants (Leibfried *et al.*, 2005). Conversely, *STM* activation results in an increase in *ARR5* expression (Jasinski *et al.*, 2005; Yanai *et al.*, 2005). Although these results appear contradictory, the induction of *ARR5* by *STM* is a consequence of its effect on cytokinin accumulation and the direct effect of *WUS*. The repression of ARRs by *WUS* thus balances its induction by cytokinin and allows differential cytokinin responses in specific regions of the meristem (Shani *et al.*, 2006).

1.6. Axillary meristem

After germination, the shoot apical meristem generates the main shoot, leaf primordia and new meristems (Aguilar-Martinez *et al.*, 2007). In the axil of each leaf, at the base of the leaf petiole, one or more secondary axillary meristem can form (Ongaro and Leyser, 2008). New shoot meristems formed in the axils of the leaves are established at the time of leaf primordial initiation or later in the development from groups of cells that retain meristematic potential (Schmitz and Theres, 2005; Aguilar-Martinez *et al.*, 2007). There are two theories about the initiation of axillary meristem. The detached meristem hypothesis proposes that axillary meristem is derived directly from cells of the SAM, which never lose their meristematic identity (Garrison, 1955; Leyser, 2003). The alternative model proposes that axillary meristem initiate *de novo* from cells in the leaf axil (Snow and Snow, 1942; Leyser, 2003). These two models have persisted because axillary meristem origin appears to be species dependent. In potato, at the time of leaf inception, meristematic cells are observed at the base of each leaf on the flanks of the primary shoot apical meristem (McDaniel and Poethig, 1988; Leyser, 2003). By contrast, in *Arabidopsis*, axillary meristems are not visible until long after leaf initiation (Gribic and Bleecker, 2000; Leyser, 2003).

During vegetative development, axillary meristems are initiated in an acropetal order, first in the axils of mature leaves distant from the shoot apex and later in younger leaves. Genes such as *LATERAL SUPPRESSOR* (*LAS*) (Greb *et al.*, 2003) or *REGULATOR OF AXILLARY MERISTEMS* (*RAX*) (Keller *et al.*, 2006; Muller *et al.*, 2006) are necessary during axillary meristem initiation to maintain the meristematic potential of leaves and to allow the organization of a stem cell niche (Aguilar-Martinez *et al.*, 2007). Other gene also involved in

early stages of axillary meristem is *REVOLUTA/INTERFASCICULAR FIBERLESS1* (*REV/IFL1*) (Talbert *et al.*, 1995).

Branching patterns depend on a key development decision: whether axillary buds grow out to give a branch or whether they remain small and dormant in the axils of the leaves. This decision is reversibly controlled by developmental and environmental stimuli (Horvath *et al.*, 2003). These environmental signals are likely to be relayed through the action of plant hormones. Of particular importance are auxin and cytokinin, as well as a new carotenoid-derived hormone (Simons *et al.*, 2007; Ongaro and Leyser, 2008).

Auxin was the first hormone linked to the regulation of shoot branching. It is known that the apex of the plant inhibits axillary bud outgrowth (Ongaro and Leyser, 2008). In pioneer experiments conducted by Thimann and Skoog (1934), they showed that auxin applied to the top of a decapitated plant mimics the effect of the removed apex, preventing bud outgrowth. Long-rang signalling promoting bud arrest is controlled both by auxin produced in the shoot apex and transported basipetally and by a novel carotenoid synthesized in the root and transported acropetally (Dun *et al.*, 2006; Aguilar-Martinez *et al.*, 2007). Mutations in the *MORE AXILLARY GROUTH* (*MAX*) genes, which control's the synthesis and activity of the carotenoid-derived hormone in *Arabidopsis* cause an excess of branch outgrowth (Booker *et al.*, 2004, 2005).

Another hormone that is involved in shoot branching is cytokinin. In contrast to the indirect inhibitory action of auxin, cytokinin directly promotes bud growth (Ongaro and Leyser, 2008). Cytokinin levels increase in buds as they activate (Emery *et al.*, 1998; Ongaro and Leyser, 2008).

Genes promoting local bud arrest within the bud have been described in monocots. They are *teosinte branched1* (*tb 1*) from maize (Doebley *et al.*, 1997) and its rice ortholog, *Os tb1* (Hu *et al.*, 2003; Takeda *et al.*, 2003). These genes are expressed in axillary meristem and buds where they suppress growth (Hubbard *et al.*, 2002; Takeda *et al.*, 2003). In 2007 Aguilar-Martinez *et al.* described *BRANCHED1* (*BRC1*) and *BRANCHED2* (*BRC2*) as the most closely related to *tb 1* in *Arabidopsis*, and showed that both genes play a central role in the control of axillary bud development.

1.7. Objectives

Previous works in our group put in evidence that *in vitro* grapevine shoots respond markedly when transferred from full nutrition medium to sulfate starvation. Responses were measured at the sulfate uptake and metabolism level and noticed as qualitative traits, namely the reversion of apical dominance inhibition triggered by routinely added cytokinin. Those traits were noticed as growth impairment and branching inhibition.

One explanation for such symptoms is the interference of sulfur deficiency with cytokinin signal pathway genes or with the expression of transcription factors acting at SAM or AM level. Therefore, and to elucidate those hypothesis, the crosstalk between cytokinin signalling and sulfur status deserved further investigation.

In the present work, *Vitis* experimental systems, namely *callus*, isolated cells and *in vitro* shoots, were used to assess the effects of sulfur deficiency through the analysis of growth parameters. As a second approach, taking advantage of *Vitis* genome sequencing, we performed a database search of genes associated to the cytokinin signal pathway and genes related with SAM and AM identity.

Finally, the expression of the former genes was analysed by quantitative real time PCR in the different biological systems under sulfur sufficient and deficient conditions.

The discussion of the results obtained so far allowed fostering tentative, certainly far from definitive, conclusions.

2. Material and methods

2.1. Plant material

Callus, cell suspensions and *in vitro* shoots of *Vitis vinifera* var. Touriga Nacional were used in all experiments.

2.1.1. Growth conditions of Vitis callus

Callus of *Vitis vinifera* cv Touriga Nacional material was maintained in the dark at 25ºC, as described in Jackson *et al.* (2001). *Circa* 5 g *callus* tissue were used as initial explant to prepare 4 *callus* pieces distributed in petri dishes with medium containing MS basal salts supplemented with 2.5 µM 2,4-D (2,4-dichlorophenoxy-acetic acid); 1 µM kinetin; 5 g l^{-1} PVP-40T; 20 g l^{-1}, sucrose; 2 g/l Gelrite at pH 5.7. The cultures growing in the dark, at 25 ºC, were sub-cultured every two weeks. Two sulfate treatments were applied: full sulfate (+S) and sulfate deprivation (-S). Commercial MS (Duchefa Biochemie, Haarlem, NL) (1.5 mM sulfate) (Murashige and Skoog, 1962) was used for +S experiments while a modified MS medium where sulfates were substituted for chlorates was considered –S. In the second sub-culture the *callus* in +S treatment were sub-culture to +S medium and –S treatment for –S medium. During the three culture cycles of two weeks, at least 4 samples per treatment were collected in each sub-culture (Fig. 5).

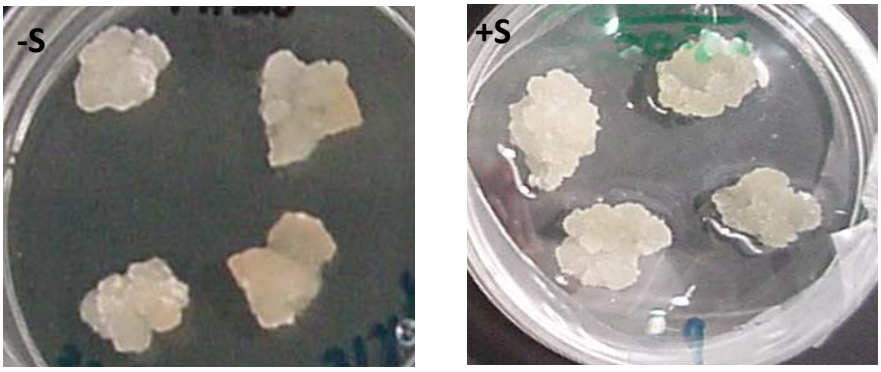

Fig. 5 *Vitis callus* in MS -S and +S medium after the first week of the first growth cycle.

2.1.2. Growth conditions of cell suspensions

Cell suspensions were obtained by adapting to liquid culture, *Vitis vinifera callus* material maintained obtained as described in ***2.1.1.***. *Ca* 4 g *callus* tissue was dispersed in 50 ml of liquid MS medium (as in ***2.1.1.)*** in 250 ml flasks. The cultures growing in a rotary shaker at 100 rpm, in the dark, at 25 ºC were sub-cultured weekly by diluting 25 ml culture into 25 ml of fresh medium. After two cycles in +S conditions two sulfate treatments were applied: full sulfate (+S) and sulfate deprivation (-S). During the 7 days culture cycle, samples of at least three flasks per treatment were collected by filtration in day 1, 4 and 7 (Fig. 6).

The growth condition for cells without cytokinin were the same as previously described, the only change was the withdrawal of cytokinin from the medium.

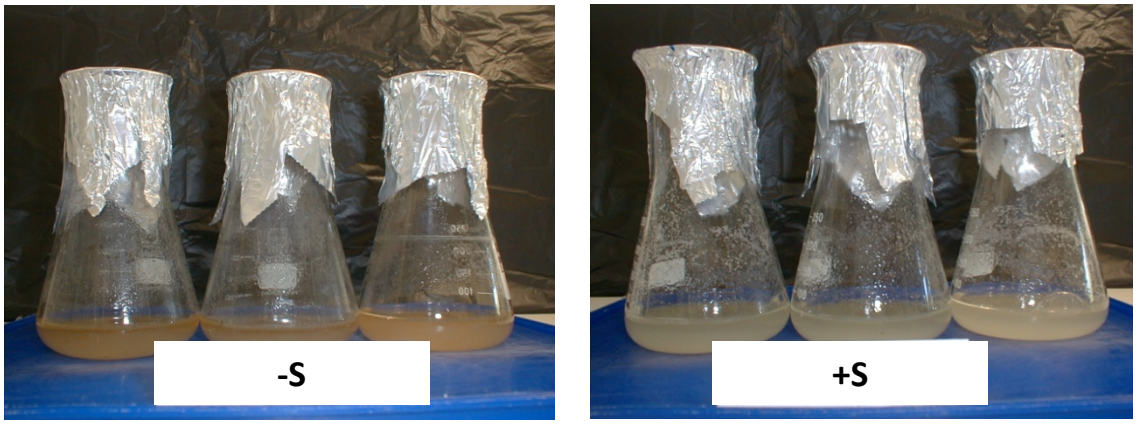

Fig. 6 *Vitis* cells growing for two weeks in a modified MS without sulfate (-S) or MS (+S) in liquid medium.

2.1.3. Growth conditions of in vitro shoots

2.1.3.1. Gel medium (GM)

In vitro shoots of *Vitis vinifera* L., var. Touriga Nacional were used as explants for *in vitro* multiplication as described in Neves *et al.* (1998). Explants were sub-cultured every four weeks into MS basal medium supplemented with 0.5 μM α-naphthaleneacetic acid (NAA), 5.0 μM 6-benzylaminopurine (BA), 30 g L^{-1} sucrose, pH 5.8, and 2 g L^{-1} Gelrite. Cultures of 5 explants in 250 mL Magenta vessels (Sigma-Aldrich St. Louis, MO) were maintained in a growth chamber under light from cool-white fluorescent lamps with 16/8h photoperiod at 50 ±

5 µmol m^{-2} s^{-1}. Temperature was 25 ± 1ºC (light) and 22 ± 1ºC (dark). For sulfate replete (+S) experiments full MS medium was used; for sulfate deprived experiments (-S) shoots were transferred to a modified MS as described above (Fig. 7). During the treatment, samples were collected in week two (40 shoots), 4 (60 shoots) and 7 (20 shoots).

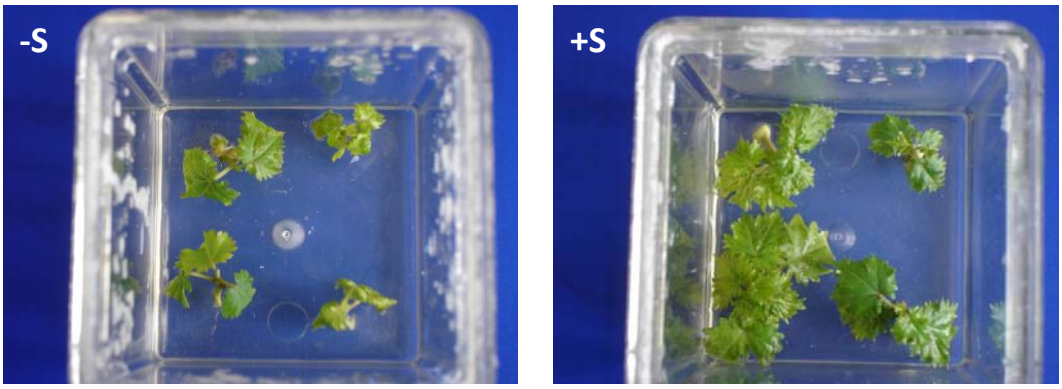

Fig. 7 Grapevine *in vitro* shoots in GM growing conditions. *In vitro* shoots were grown for 4 weeks in a modified MS medium without sulfate (-S) or MS (+S).

2.1.3.2. Temporary immersion system (TIS)

In vitro shoots of *Vitis vinifera* L., obtained as in ***2.1.3.1.*** were used as explants for *in vitro* multiplication in Temporary Immersion System (TIS) in the same medium composition (without Gelrite) and physical conditions. TIS cultures were performed in pairs of 500 mL flasks (Schott, Duran) one containing 15 shoots and the other 300 mL of liquid medium. After two cycles of multiplication in +S conditions the shoots were sub-cultured for elongation medium for one week, and then to root expression medium for two weeks. This system enables the contact between all parts of the explant with the liquid medium. A pumping system transfers the medium from one flask to the other holding the shoots, which were immersed for 4 min every three hours (Fig. 8). During the treatment, samples were collected in week two (15 plantlets) and week three (15 plantlets).

Fig. 8 Temporary Immersion System (TIS). Grapevine *in vitro* shoots were grown for three weeks in a modified MS medium without sulfate (-S) or MS (+S).

2.2. Determination of growth parameters

2.2.1. Biomass of callus and cells

Several growth parameters were register at the 2nd, 4rd and 6th weeks, for *callus*, and 1st, 3rd, 4th, 6th and 7th days of culture, for cells: fresh weight (FW) and dry weight (DW). The Relative Growth Rate (RGR) calculated as $(W_f - W_i) / W_i$, where W_f is the final dry weight at 2nd, 3rd week of culture and W_i is the initial dry weight at day 0.

2.2.2. Growth parameters of in vitro shoots

At the 2nd, 3rd week of culture several growth parameters were registered: FW, DW and RGR as $(W_f - W_i) / W_i$, where W_f is the final dry weight at 2nd, 3rd week of culture and W_i is the initial dry weight at day 0, number of new branches formed *in vitro* and number of new leaves.

2.3. Total chlorophyll and chlorophyll a/b ratio

Extraction of total chlorophyll was carried out using 4 leaf disks (total area 113.2 mm^2) and 3 ml of Dimethyl sulfoxide (DMSO) pre-heated to 65 ºC for one hour, according with the method described by Hiscox *et al.* (1978). Optical density (OD) was measured at 645 and 663 nm against DMSO. The chlorophyll content of the extracts was using the equations described by Porra *et al.* (1989).

$$\text{Chl}a\ (\mu g\ mL^{-1}) = 12{,}00\ X\ A_{663} - 3.11\ x\ A_{645}$$

$$\text{Chl}b\ (\mu g\ mL^{-1}) = 20{,}78\ x\ A_{645} - 4{,}88\ x\ A_{663}$$

$$\text{Tot Chl}\ (\mu g\ mL^{-1}) = 17{,}67\ x\ A_{645} + 7{,}12\ x\ A_{663}$$

These results were converted to mg Chl cm^{-2} leaf area (Richardson *et al.*, 2002). For this analysis explants from GM and TIS were used.

2.4. Sequence retrieval and database search

Multiple database searches in NCBI (http://www.ncbi.nlm.nih.gov/) and Genoscope (http://www.genoscope.cns.fr/spip/), were performed to identify members of the cytokinin signalling pathway in *Vitis*.

To detect putative genes involved in the *Vitis* cytokinin two-component signalling, *Arabidopsis* protein sequences AHK2, AHK4, AHK3, AHP3 and ARRs (A-type RR17 and another for B-type RR18), WUS, CLV and STM for apical meristem and BRC1, BRC2, LAS, RAX and REV for axillary meristem, acquired from the NCBI databases were used as queries and then re-searched using *Vitis* proteins as queries. The phylogenetic trees were constructed using the PHYLIP, PRODIST and NEIGHBOR programs (Felsenstein, 2005).

After obtaining the protein sequence for each hit, the same database was used to obtain the full-length cDNAs of all predicted genes.

2.5. Gene expression

2.5.1. RNA extraction and cDNA preparation

Total RNA was extracted from *Vitis* cells of using RNeasy plant Mini Kit (Qiagen, Hilden, Germany). All RNA samples were treated with RNase free DNase I (Qiagen, Hilden, Germany) according to the manufacturer protocol and quantified using absorption of U.V. light at 260 nm. Reverse transcription was carried out using superscript III RNase H- reverse transcriptase priming with oligo-d(T) (Invitrogen) according to the manufacturer's recommendations.

Total RNA was extracted from *Vitis* shoots using the method described by Reid *et al.* (2006). Tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was added to pre-warmed (65°C) extraction buffer (300 mM Tris HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% CTAB, 2% PVPP, 0.05% spermidine trihydrochloride, and just prior to use, 2% β-mercaptoethanol) at 20 ml per g of tissue and shaken vigorously. Tubes were

subsequently incubated in a 65°C water bath for 10 min and shaken every two min. Mixtures were extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1) then centrifuged at 3,500 g, 15 min at 4°C. The aqueous layer was transferred to a new tube and centrifuged at 30,000 g, 20 min at 4°C to remove any remaining insoluble material. To the supernatant, 0.1 vol 3 M NaOAc (pH 5.2) and 0.6 vol isopropanol were added, mixed, and then stored at -80°C for 30 min. Nucleic acid pellets (including any remaining carbohydrates) were collected by centrifugation at 3,500 g, 30 min at 4°C. The pellet was dissolved in 0,5 ml miliQ H_2O and transferred to a microcentrifuge tube. To selectively precipitate the RNA, 0.3 vol of 8 M LiCl was added and the sample was stored overnight at 4°C. RNA was pelleted by centrifugation at 20,000 g, 30 min at 4°C then washed with ice cold 70% EtOH, air dried, and dissolved in 50µl DEPC-treated water. All RNA samples were treated with RNase free DNase I (Qiagen) according to the manufacturer protocol. Quantification and reverse transcription was carried out using the same protocol as described for *Vitis* cells.

Table I Real-time PCR primers designed from *Arabidopsis* sequences and validated with *Vitis* genome.

Arabidopsis protein	Initial Seq. from data base (*Vitis*)	Name	Primer sequence	Sequences tested by Real-Time PCR
HK's				
AHK2	CU459264	VvCyt1	5' GAAGTGCTGAGACAGAGCTTGAATA 3' 5' CTCCATTGAATCTGTGCAGCTTAAC 3'	+
CRE1	CU459222	VvCyt2	5' ATTCGAGACGAGTATGCACCTGTGA 3' 5' ATGATGAGAACCAAGCAGCCTGAAG 3'	+
AHK3	CU459353	VvCyt3	5' TAGCTGCTGGTGCATTGAAGAAGTA 3' 5' TCGACTATTGACGTTCCGTTCCATT 3'	+
AHP3				
	CU459291	VvHP1	5' CTGGCGGAGATAATACCAATGT 3' 5' ATGTATCTCGCATTACCTGAAC 3'	
	CU459224	VvHP2	5' AATTCGTTCAGCTGGAGGAACT 3' 5' TTCAGCTGTTGGAAGGTCTTCA 3'	+
	AM484268	VvHP3	5' CACAGCTTCAGCAACTACAAGA 3' 5' TTGTTGCTCCAACCTGAAGAGA 3'	+
	CU459321	VvHP4	5' GAATCTCCGAGGACTACTGATG 3' 5' AACAGTACTCGTGTTCGACAAG 3'	
A-type RR17				
	CU459263	VvRRa1	5´ TGTATGCCTGGAATGACTGGAT 3' 5' TGGACGGTGGTGATGATGATAA 3'	
	CU459229	VvRRa2	5' TTGGGTTCCTCCACCACTGTAT 3' 5' ACCTTCCGATCCACATGGCTAT 3'	
	CU459265	VvRRa3	5' AAGGAGGTTCCAGTTGTGATAA 3' 5' GCTTCTGTGCTTCAAGTAACAT 3'	+
	CU459270	VvRRa4	5' ACTGGCTATGATCTCCTCAGAA 3' 5' GCTTGCTCACATCCGATAATTG 3'	+

Table I Real-time PCR primers designed from *Arabidopsis* sequences and validated with *Vitis* genome (Cont.).

Arabidopsis protein	Initial Seq. from data base (*Vitis*)	Name	Primer sequence	Sequences tested by Real-Time PCR
B-type RR18				
	AM434392	VvRRb1	5' CAGACGTGGAATGAAGTCCTAA 3' 5'TCTGAGGTCTCTGGATCTACAA 3'	
	AM432245	VvRRb2	5' AACCAAGATTCCCTCGGCAACCT 3' 5' TACCTGCACCGTTGGCTTGATA 3'	
	AM423607	VvRRb3	5' GATTACTCATGGTGCTTGTGAC 3' 5' CTCATTCTCTTCTCCGTTCTCT 3'	
	AM460059	VvRRb4	5' AGTAACCTTGATCCGAGTAGAA 3' 5' CTGCTGGAACAACATATCTTGA 3'	
	CU459292	VvRRb5	5' TTCGAGTTCTCGTGGTCGATGA 3' 5' CATGCACCATGCTGAACACCTT 3'	+
	CU459281	VvRRb6	5' TCCTGGTGGTTGATGATGATCC 3' 5' TGATAACAGGCAGGTCCATCTC 3'	+
Apical Meristem genes	CU459300	VvWus	5' CTGGACTCCTACAACTGACCAGATA 3' 5' TTCTTGCCTTCGATCTTGCCGTACT 3'	+
	CU459218	VvClv	5' TTCGGTTGTCGAACCTCCAAGGATT 3' 5' AAGTCAAGAACTCGGAGCGAACTCA 3'	+
	AM483920	VvSTM	5' TCTATGGTGATGATGATGCCTCCTA 3' 5' ATTGTTGCTGCTGTTGTTGTTGTTC 3'	+
Axillary Meristem genes	CU459233	VvBrc1	5' TCTTCCTTCTTCTACTTTCCGTCTC 3' 5' TTGCGATCTCCTATTAGTTCATTGC 3'	+
	CU459222	VvBrc2	5' TGCCCGTAAGTTCTTTGATCTCCA 3' 5' GGTGAGTTCCTTGATTGCTGCTTTG 3'	+
	CU459359	VvLas	5' GAGCCATGTCTGCTAGTGCTAATAC 3' 5' CCAGAATAGCTTGATTGGCAGTGAG 3'	+
	CU460733	VvRax	5' GAGCTAACTACGTCAAGCAAGAGAT 3' 5' ATCTTGGTTCGGATGCACAGATACT 3'	+
	CU459220	VvRev	5' AAGGCTACAGGAACTGCTGTCGATT 3' 5' TGCCACTCCACTGCAACTATGTGAA 3'	+

To examine gene expression, specific primer pairs were designed (Table I), using the Clone Manager program (Scientific & Educational Software, NC, USA). The primers were designed to amplify fragments between 100 and 200 bp, so that they could be used for real-time PCR, except a primer pair designed to amplify a larger product of *VvCyt1* (750 bp). To confirm the amplification of the targeted genes, a PCR reaction was conducted in a Master Cycler Gradient thermocycler from Eppendorf, under the following conditions: 95 ºC, 3 min (initial polymerase activation), 94 ºC, 3 min; 40 cycles at 95 ºC, 15 s (denaturation); 61 ºC, 30 s (annealing); 72 ºC, 20 s (extension). PCR products were resolved on 2% (w/v) agarose gels run at 4 V cm^{-1} in Tris-acetate-EDTA buffer (TAE), along with a 1Kb plus DNA ladder (Invitrogen Gmb H) to confirm the presence of a single product of the desired length.

2.5.2. Purification and sequencing of DNA fragments

The PCR products obtain in **2.5.1.** were purified using the Wizard® SV Gel and PCR Clean up system (Promega, Madison, USA). The fragments were then sequenced. The sequencings were performed by STABVIDA (Oeiras, Portugal) and Macrogen (Seoul, Korea). The sequence alignments were then carried out in BioEdit program, and their identity confirmed by searches in NCBI database, using the translated protein product. Nucleotide sequences of cDNAs are presented in supplementary data.

2.5.3. Gene expression analysis by Real-Time PCR

Real-time PCR was performed in 20 µL of reaction mixture composed of cDNA, 0.5 µM gene-specific primers and master mix iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using an iQ5 Real Time PCR (BioRad). Reactions conditions for thermal cycling were: 95º C for 3 min; 40 cycles of 95ºC for 10 sec; 61º C for 25 sec; 72º C for 30 sec and 71 cycles of 60º C for 30 sec to obtain the melt curve. The detection of PCR product was monitored by measuring the fluorescence after each extension step caused by the binding of SYBR green dye to dsDNA. Each run was completed with a melting curve analysis to confirm the specificity of amplification and confirm the absence of primer dimmers. Relative amounts were calculated and normalized with respect to Actin (Act) mRNA levels. Data were analyzed with the iQ5 optical system software (Bio-Rad, Hercules, CA), which calculates the threshold cycle (C_T) and exported into a MS Excel workbook (Microsoft Inc.) for further analysis. Each reaction was done in triplicate and corresponding C_T values were determined. The method described by Bovy *et al.* (2002) was applied to compare the level of gene expression in –S to +S conditions.

First, the C_T values were normalized to the C_T value of Act2 (An *et al.*, 1996), a housekeeping gene found to be expressed at constant level in the conditions tested. Next, the expression level of each gene in –S condition was expressed relative to its expression in +S conditions according to the equation:

$$\Delta\Delta C_{T(+S/-S)} = \Delta C_{T(-S)} - \Delta C_{T(+S)}$$

Finally, the relative expression of the genes in - S conditions was expressed according to the expression

$$2 \exp \Delta\Delta C_{T(+S/-S)}$$

where exp=exponential.

2.6. Statistics

For each parameter, S treatment and control were performed at least twice. Data are presented as mean values ± standard deviation (SD).The results were statistically evaluated by variance analysis (ANOVA) comparing the two treatments applied in our study (+S and –S) using MS Excel workbook (Microsoft Inc.) software and Statistica 8 (Statsoft Inc.).

3. Results

3.1. Physiology of experimental systems under sulfate deficiency

The measurement of physiological parameters is a valuable mean to assess whether imposed conditions affect the functioning of the biological systems including model experimental systems. Our main aim was to determine if the cytokinin signalling pathway was affected, in grapevine (*Vitis vinifera* L.) by sulfate deficiency. For that purpose different experimental models were used: *Vitis callus*, *Vitis* plantlets in gel medium and *Vitis* plantlets in Temporary Immersion System (TIS). Cell cultures were also used in cytokinin withdrawal experiments. The effect of sulfate nutrition on cell growth as previously analyzed (Tavares *et al*., 2008) is not included in the present study.

3.1.1. Growth of Vitis callus

The growth analysis of plant systems is an unequivocal way to assess the effect of any stressful situation. In the present study, the effect of sulfur depletion on the growth of *Vitis callus* was quantified.

The fresh weight (FW) of *Vitis callus* grown in full MS culture medium (+S) and a modified MS medium without sulfate (–S) along three cycles of two weeks is showed in Fig. 9. The initial fresh weight of each cycle is *ca* the FW at time 0. At the end of the each cycle +S *Vitis callus* almost doubled the FW when compared with that at time 0. After the second cycle the FW was significantly affected by sulfur deficiency and, at the fourth week the FW of -S *Vitis callus* was *ca* 50% the value of +S *callus*, and even less after 6 weeks in –S conditions. No further growth cycles were attempted due to growth inhibition and also to oxidation symptoms of the tissues (Fig. 5).

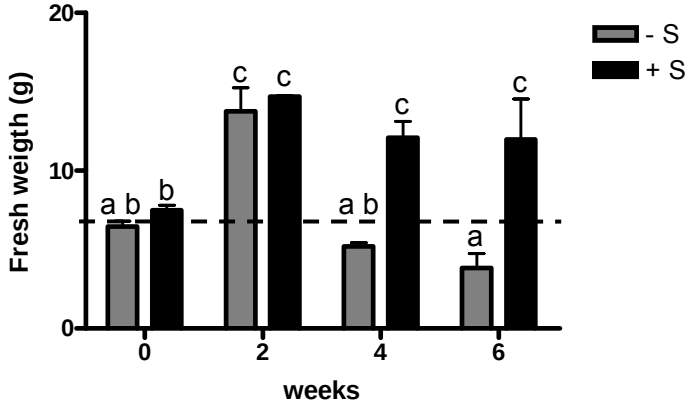

Fig. 9 Fresh weigth of *Vitis callus* in deficient (–S) and replete (+S) conditions grown for 2, 4 and 6 weeks. Bars represent means of the FW of 4 *Vitis callus* taken randomly ± SD. **---** represents the FW of *callus* at installation of each cycle. Different letters indicate significant differences at $p<0.05$.

FW combines water content and dry matter. A way of measuring the relative content of water or dry matter is through the ratio between dry weight and fresh weight (DW/FW).

In Table II we can notice that –S *callus* increase of DW/FW ratio, from the 2nd to the 6th week, what indicates that *Vitis callus* in –S medium produces more dry matter (Table II).

Table II Dw/Fw ratio of *Vitis callus* in +S and –S growing conditions. *Vitis callus* were grown for 2, 4 and 6 weeks in MS medium with sulfate (+S) and without sulfate (-S).

	Dw/Fw		
	2 weeks	4 weeks	6 weeks
+S	0,030	0,028	0,034
-S	0,032	0,041	0,048

For characterizing *callus* growth, Relative Growth Rate (RGR) is one useful parameter. As expected, the RGR of *Vitis callus* grown in +S did not change significantly. After two weeks in –S medium, no significantly different RGR was noticed while at the fourth (second cycle) and sixth week (third cycle) the RGR was negative (Fig. 10).

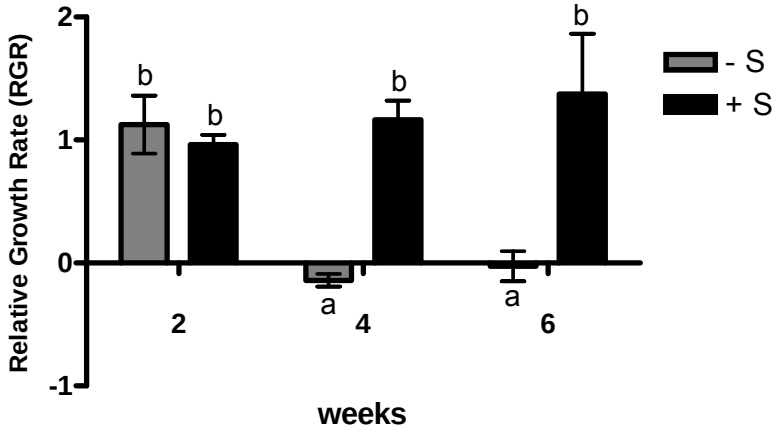

Fig. 10 Relative Growth Rate (RGR) of *Vitis callus* in deficient (–S) and replete (+S) sulfate conditions grown for 2, 4 and 6 weeks. Bars represent means of 4 *callus* samples taken randomly ± SD. Different letters indicate significant differences at $p<0.05$.

3.1.2. Growth of Vitis cells in cytokinin deficiency

Cell biomass was used to assess the effect of cytokinin on the growth of *Vitis* cells.
The FW of *Vitis* cells grown for 7 days in MS medium with or without cytokinin is presented in Fig. 11. The biomass of *Vitis* cells was not significantly affect by cytokinin depletion during de first 5 days of treatment, only in day 7 the FW of cells without cytokinin was lower than in control cells (Fig.11).

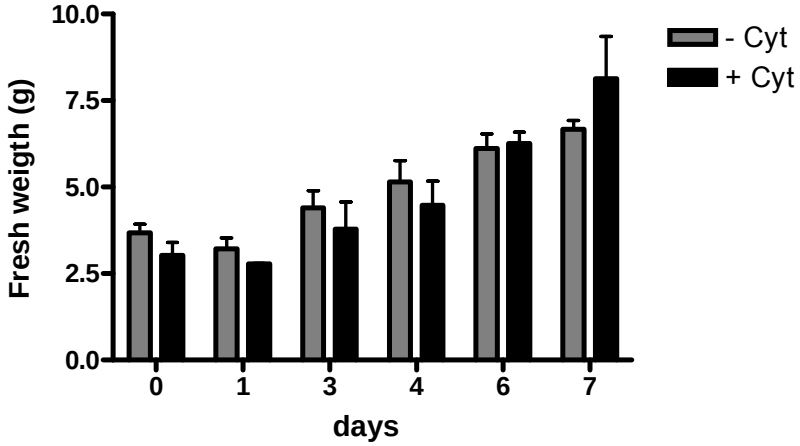

Fig. 11 Fresh weight of *Vitis* cells grown for 7 days in replete (+S) MS medium in the absence (-Cyt) or presence (+Cyt) of cytokinin. Bars represent means of the FW taken randomly ± SD.

3.1.3. Growth of in vitro shoots

3.1.3.1. In gel medium (GM)

3.1.3.1.1. Fresh Weight and Relative Growth Rate

The FW of grapevine shoots grown in +S and –S MS gel medium along a 4 weeks culture cycle and after the transfer of –S plantlets for more two weeks into –S medium, is presented in Fig. 12 while Fig. 13 represents the RGR obtained at the same time points. The FW of shoots was significantly affected by the sulfur deficient conditions: +S shoots doubled in FW after 4 weeks when compared to time 0, while -S *in vitro* shoots showed no increase in FW in any of the reported time points (Fig. 12). Consequently RGR of –S Shoots was not far from null (Fig. 13).

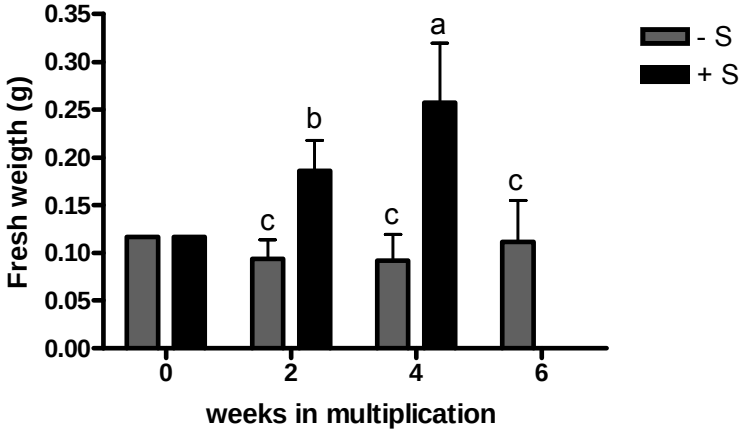

Fig. 12 Fresh weight of grapevine *in vitro* shoots in –S and +S growing conditions. *In vitro* shoots were grown for 2, 4 and 6 weeks in medium without (-S) and with (+S) sulfur. Each bar is the mean of the FW of ten *in vitro* shoots ± SD. Different letters indicate significant differences at $p<0.05$.

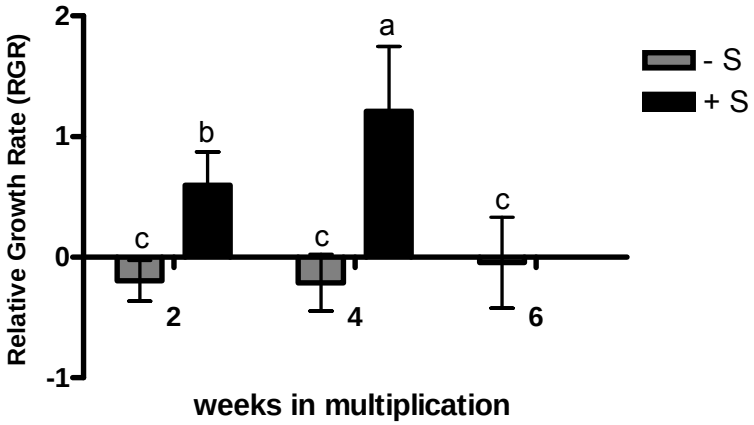

Fig. 13 Relative Growth Rate (RGR) of *in vitro* shoots in deficient (–S) and replete (+S) multiplication conditions grown for 2, 4 and 6 weeks. Bars represent means of 40 (2nd week), 60 (4th week) and 20 (6th week) samples taken randomly ± SD. Different letters indicate significant differences at $p<0.05$.

3.1.3.1.2. Branching

The raised branching, verified in *in vitro* multiplication, is based on the inhibition of apical dominance by exogenous cytokinin addition. The modification of the branching rate by – S nutrition could result from an effect of cytokinin signal transduction. We verified that the multiplication rate of +S shoots increased along the 4 weeks culture cycle , while the number of shoots maintained constant in –S shoots (Fig. 14).

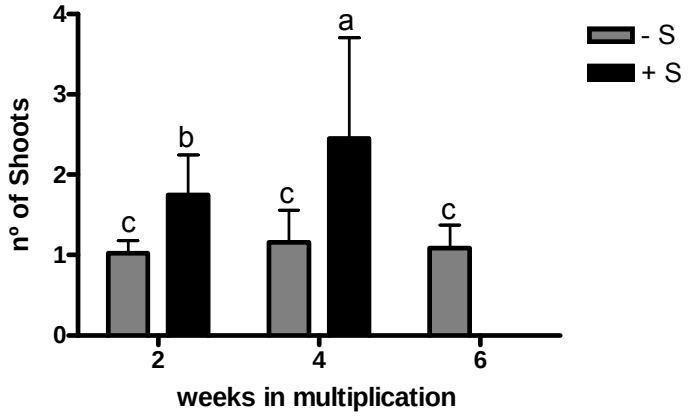

Fig. 14 Number of shoots produced by one single node of *in vitro* shoots grown for 2, 4 and 6 weeks in deficient (–S) and replete (+S) multiplication conditions. Bars represent means of 40 (2nd week), 60 (4th week) and 20 (6th week) samples taken randomly from two independent experiments ± SD. Different letters indicate significant differences at $p<0.05$.

The number of leaves was also affected by the –S conditions of the medium. +S conditions led to a higher number o leaves per shoot, especially because a higher number of branches were observed in this condition. However the number of leaves produced per branch was similar in –S and +S media, with a slight higher value in the last medium (results not shown).

3.1.3.1.3. Total chlorophyll and chlorophyll a/b ratio

As expected, the total chlorophyll content of +S *V. vinifera* shoots did not change during the 4 weeks of multiplication but the –S conditions affected significantly the total chlorophyll content (Fig. 15), which decreased along the 6 weeks multiplication period. After 6 weeks in –S medium the shoots presented visible symptoms of chlorosis.

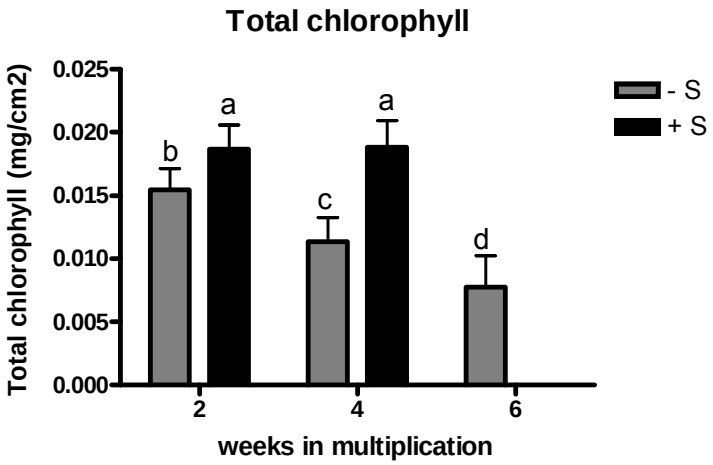

Fig. 15 Total chlorophyll (mg cm^{-2}) in leaves of *in vitro* shoots grown for 2, 4 and 6 weeks in deficient (–S) and replete (+S) multiplication conditions. Bars represent means of 10 samples taken randomly ± SD. Different letters indicate significant differences at $p<0.05$.

The chlorophyll a/b ratio presented similar values in the two conditions studied, so the decrease of total chlorophyll must indicate a slight tendency for a higher decrease in chlorophyll b (Fig. 16).

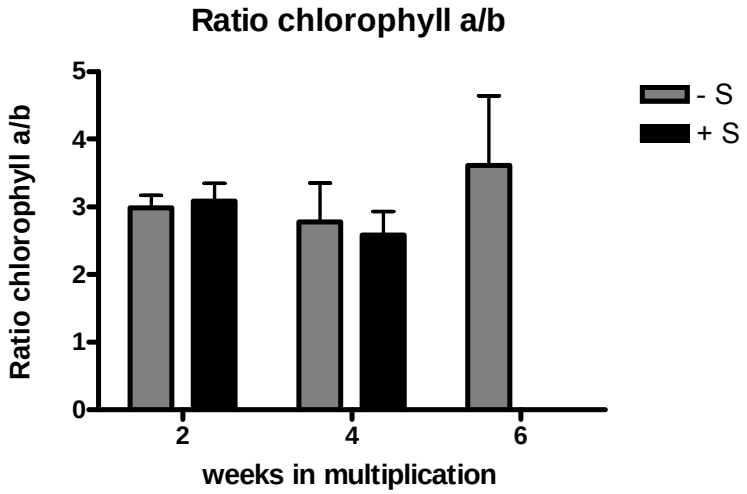

Fig. 16 Chlorophyll a/b ratio in leaves of *in vitro* shoots grown for 2, 4 and 6 weeks in deficient (–S) and replete (+S) multiplication conditions. Bars represent means of 10 samples taken randomly ± SD.

3.1.3.2. In Temporary Immersion System (TIS)

The use of TIS has proven to be an alternative to GM as an efficient propagation technique in what concerns plantlet morphology and physiology. The renovation of head-space atmosphere approaches TIS of autotrophic conditions. So the TIS was chosen to study the effect of the absence of sulfate in growth condition more close to autotrophic growth.

3.1.3.2.1. Branching

The –S multiplication medium affected drastically the multiplication rate of the *in vitro* shoots. After two weeks in +S multiplication medium *V. vinifera* shoots tripled the initial number of branches, while –S only doubled (Fig. 17) with an average of two branches per shoot. After three weeks in +S the majority of the plantlets (new roots start protruding at this time point) showed three or more growing buds, with an average of 5 new branches per plantlet, while in –S plantlets the highest number of growing buds was 4, with an average number of three, and very small in size (Fig. 17).

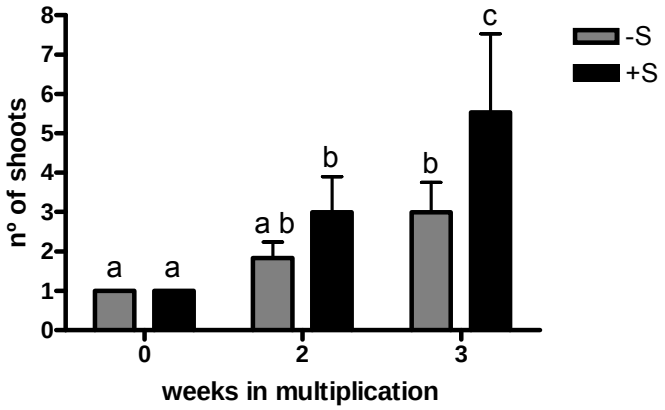

Fig. 17 Number of shoots produced by one single node of *in vitro* shoots grown for 0, 2 and 3 weeks in deficient (–S) and replete (+S) multiplication conditions. Bars represent means of 30 (1st week), 6 (2nd week) and 15 (3rd week) samples taken randomly ± SD. Different letters indicate significant differences at $p<0.05$.

In TIS culture, –S shoots started to show symptoms of chlorosis, after only two weeks in culture. The chlorosis was the result of an alteration of total chlorophyll in the plantlet (Fig. 18).

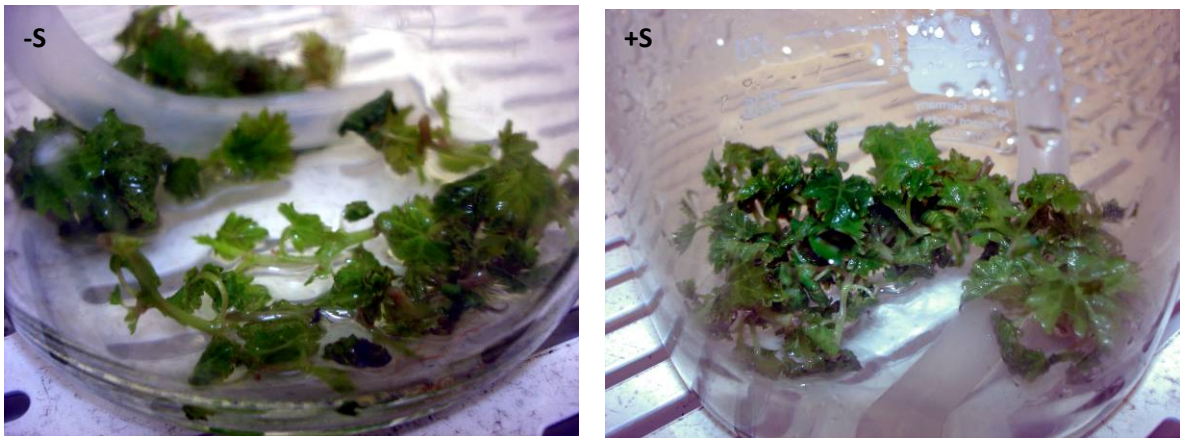

Fig. 18 *V. vinifera* shoots grown for two weeks in deficient (–S) and full (+S) sulfate conditions in TIS, (n=15 shoots).

3.1.3.2.2. Total chlorophyll and chlorophyll a/b ratio

In TIS the total chlorophyll content of +S shoots increased slightly at the third week of multiplication. The –S conditions affected significantly the total chlorophyll content, decreasing at the second week and attaining a value not higher than 30% the +S at the third week (Fig. 19). After two weeks in –S medium the shoots showed already visible symptoms of chlorosis. (Fig. 18)

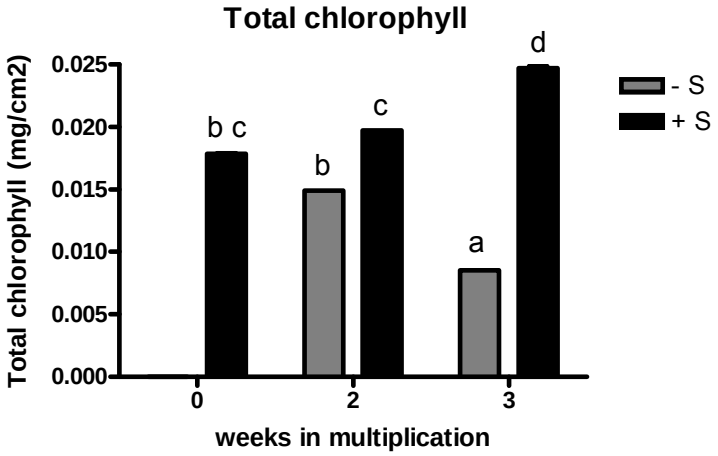

Fig. 19 Total chlorophyll (mg cm^{-2}) in leaves of *in vitro* shoots in TIS grown for three weeks in sulfate deficient (–S) and replete (+S) multiplication conditions ± SD. Different letters indicate significant differences at $p<0.05$.

The chlorophyll a/b ratio decreased in –S plantlets along the multiplication cycle, possibly due to a higher decrease in chlorophyll a (Fig. 20).

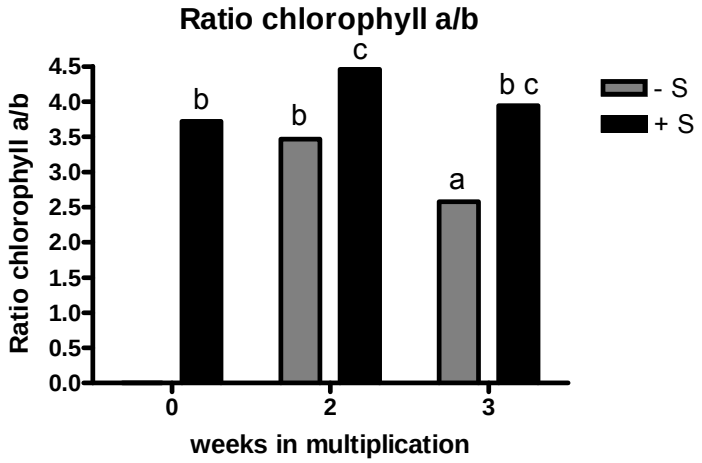

Fig. 20 Ratio chlorophyll a/b in leaves of in vitro shoots grown for three weeks in sulfate deficient (-S) and replete (+S) multiplication conditions. Different letters indicate significant differences at $p<0.05$.

3.2. Identification and characterization of genes associated with cytokinin signalling

Sulfur affects plant by a decreasing its growth. Cytokinin is an essential plant hormone that controls various aspects of plant including plant growth. The effect of sulfur may be caused by a disruption in cytokinin signal pathway or an alteration of apical and axillary meristems.

The sequencing of the *Vitis* genome allowed a molecular analysis of the genes associated to the former mechanisms.

Our first approach was to evaluate the phylogenetic relationship between *Vitis* cytokinin signalling genes and the annotated genes in other species. For that purpose we performed phylogenetic analysis by comparing different sequences through alignment techniques

3.2.1. Cytokinin receptor (HK)

Using the cytokinin receptors annotated in *Arabidopsis* and deposited at NCBI Database, AHK2, CRE1/AHK4, and AHK3, as initial queries, we performed a database search and identified three Histidine Kinases (HK) genes in *Vitis* genome, VvCyt1, VvCyt2 and VvCyt3. As the cytokinin receptors described so far, the VvCyt genes encode proteins with a CHASE domain, a Histidine Kinase A domain, a Histidine kinase-like ATPase domain and a signal receiver domain (Fig. 2). The phylogenetic analysis of the amino acid sequences identified a high degree of similarity to *Arabidipsis*, maize and rice cytokinin signalling HKs. More specifically, VvCyt1 shares 65% identity with AHK2, VvCyt2 is similar to CRE1/AHK4 in 69% of the aminoacids and VvCyt3 has a high correlation with AHK3, sharing 68% homology, and with HKs from *Lupinus* and maize. Therefore, there is a strong probability that these proteins correspond to cytokinin HK receptors in *Vitis* (Fig. 21).

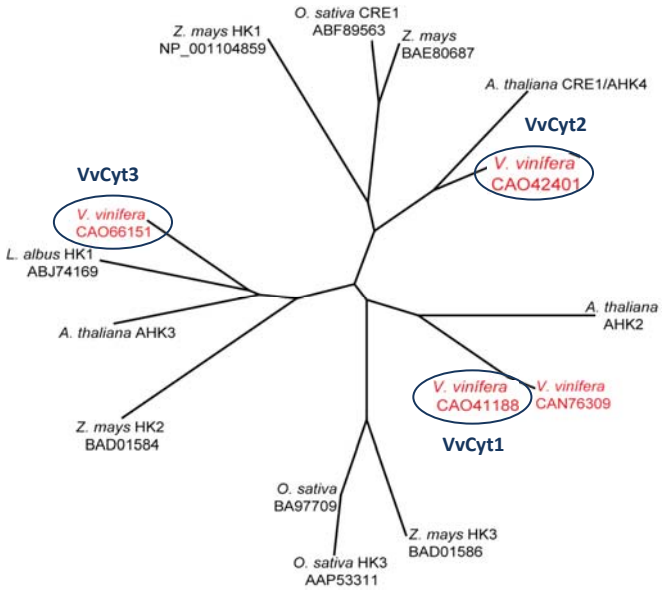

Fig. 21 Phylogenetic tree for HKs. The circled assignments correspond to sequences analyzed by real time PCR for Vitis cells gene expression. The phylogenetic tree was constructed using the PHYLIP programs, PRODIST and NEIGHBOR (Felsenstein, 2005).

3.2.2. Histidine-containing phosphotranmitter (HPt)

A database search using the *Arabidipsis* Histidine-containing phosphotransmitter protein, AHP3, as initial query, four HPt genes were identified in *Vitis*, but only two (VvHP2 and VvHP3) were used for further studies. VvHPs encode proteins with a conserved Histidine Phosphotransfer domain (Fig. 2), involved in signalling through a two part component systems in which an autophosphorylating histidine protein kinase serves as a phosphoryl donor to a response regulator protein. The amino acid sequence of VvHP3 shares 59% identity with *Arabidopsis* AHP1 while VvHP2 shares 72% homology with *Arabidopsis* AHP4 and is closer to some rice sequences, (Fig. 22).

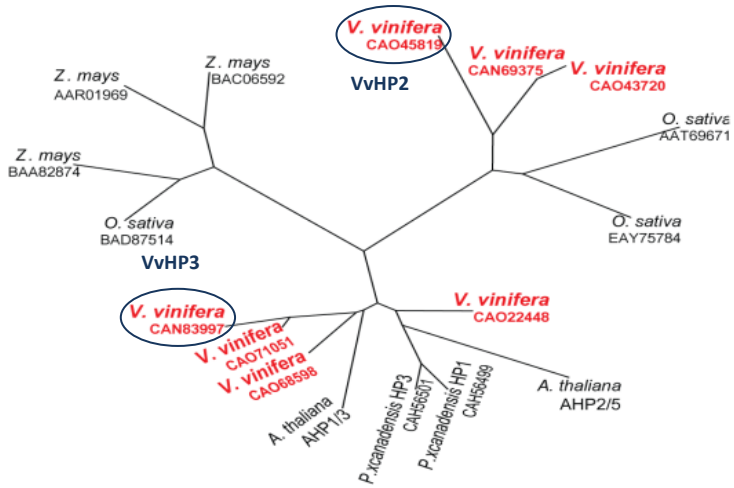

Fig. 22 Phylogenetic tree for HPs. The circled assignments correspond to sequences analyzed by real time PCR for Vitis cells gene expression. The phylogenetic tree was constructed using the PHYLIP programs, PRODIST and NEIGHBOR (Felsenstein, 2005).

3.2.3. A-type response regulator (RRa)

A database search using *Arabidopsis* ARRa17 protein as query, identified four *Vitis* RRas aminoacid sequences corresponding to the coding genes, from which two (VvRRa3 and VvRRa4) were used for further studies. VvRRas encode a signal receiver domain, known as REC, which receives the signal from the sensor partner (HPt) in a two-component system mechanism (Fig. 2). The aminoacid sequence of VvRRa3 shares 74% identity with ARRa17 and 67% identity to ARRa16, while VvRRa4 is closer to rice RRa9 and *Z. mays* RRa6 (72% and 67% identity, respectively) (Fig. 23).

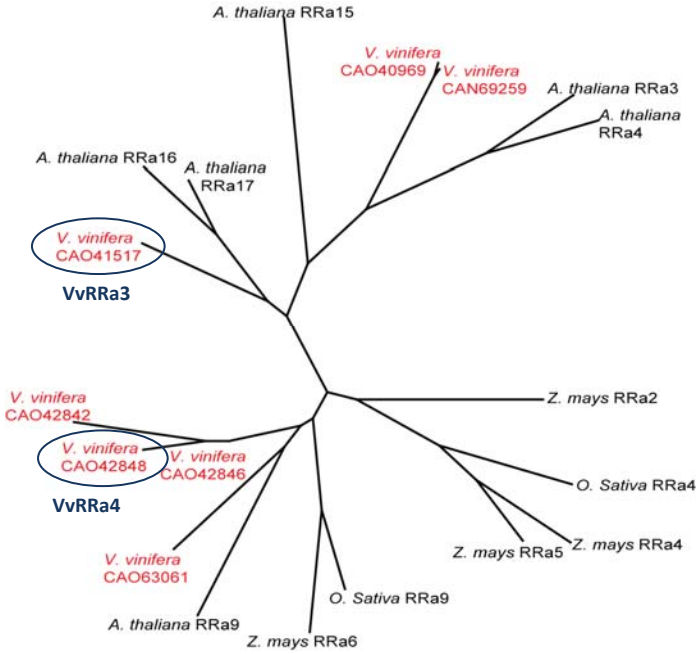

Fig. 23 Phylogenetic tree for RRa. The circled assignments correspond to sequences analyzed by real time PCR for Vitis cells gene expression. The phylogenetic tree was constructed using the PHYLIP programs, PRODIST and NEIGHBOR (Felsenstein, 2005).

3.2.4. B-type response regulator (RRb)

A database search using *Arabidopsis* ARRb18 protein as query identified four RRbs genes in *Vitis*, but only two (VvRRb5 and VvRRb6) were used for further studies. B-type RRs encode a signal receiver domain which, as the A-type RRs, receives the signal from the sensor partner (HPt) in a two-component system mechanism (Fig. 2). The aminoacid sequence of VvRRb5 is closer to ARRb11, with an identity of 56%, while VvRRb6 is closer to ARR18 and *Z. mays* and rice RRb9, with a homology of 45% (Fig. 24).

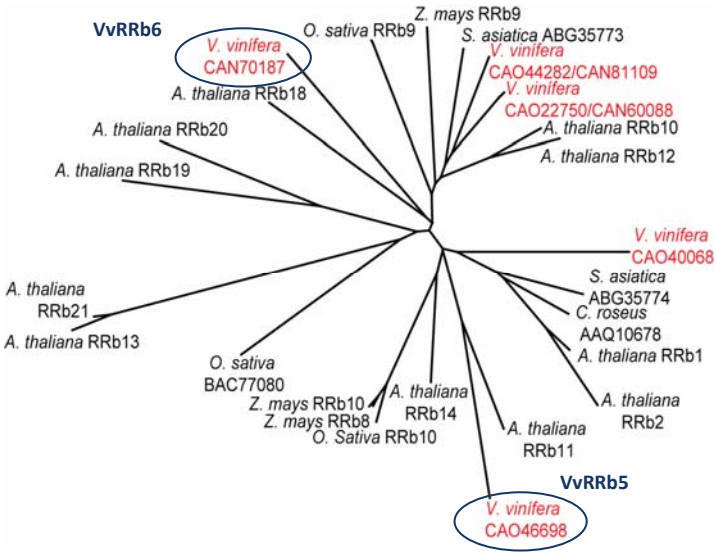

Fig. 24 Phylogenetic tree for RRb. The circled assignments correspond to sequences analyzed by real time PCR for Vitis cells gene expression. The phylogenetic tree was constructed using the PHYLIP programs, PRODIST and NEIGHBOR (Felsenstein, 2005).

3.3. Analysis of RT-PCR products

3.3.1. Cytokinin signalling genes *in Vitis cells*

For this point of the study cell suspensions were selected in order to deal with a homogeneous experimental system well adapted to molecular analysis, where sulfur and cytokinin manipulation in short periods was easy to achieve in short periods.

The amplification of transcripts of *Vitis* cytokinin signalling genes with specific primers was confirmed by PCR analysis. The products were resolved on agarose gels (percentage of agarose (w/v) indicated in the legends) for confirming its presence in the cells suspension. When the presence of predicted fragments was identified, these fragments were then sequenced.

The analyses of the gels revealed that primers designed for *Vitis* cytokinin receptors amplified fragments of the expected size, confirming the expression of cytokinin receptors in *Vitis* cells. As expected, primers designed for a conserved region of *VvCyt1* (FJ822975) amplified a fragment of 751 bp while the primers designed for *VvCyt2* and *VvCyt3* (FJ822976) amplified fragments of 127 bp and 190 bp, respectively (Fig. 25)

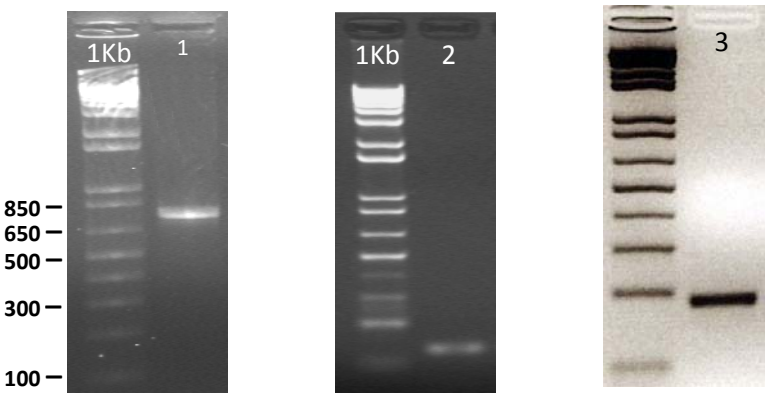

Fig. 25 PCR products from *Vitis* cells resolved on 1.5 % (W/V) agarose gel, 1) *VvCyt1*, 2) *VvCyt2* and 3) *VvCyt3*.

In the PCR preformed with primers designed for *VvHP2* (FJ822977), *VvHP3* (FJ822978) and *VvHP4* (FJ822979) we could verify the presence of the predicted fragments with 293 bp, 309 bp and 208 bp, respectively (Fig. 26). Only the primers designed for *VvHP1* never amplified any fragment. It is reasonable to assume that *VvHP1* is not expressed in *Vitis* isolated cells.

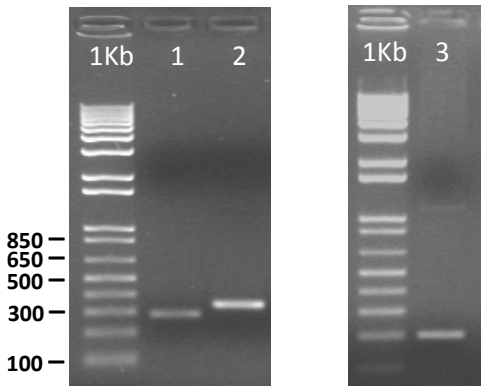

Fig. 26 HP PCR products resolved on 1% (w/v) agarose gels. 1) *VvHP2*, 2) *VvHP3*, 3) *VvHP4*.

The analyses of RR PCR products allowed to identify the presence of the predicted products with all primers pairs. The amplification of *VvRRa3* and *VvRRa4* showed more fragments than expected (Fig. 27 A), which, in a latter Real-Time PCR analysis, did not cause any interference. Primers designed for *VvRRa1* (FJ822980), *VvRRa2* (FJ822981), *VvRRa3* (FJ822982) and *VvRRa4* (FJ822983) amplified fragments with 328 bp, 199 bp, 150 bp, 169 bp, respectively. In the PCR preformed with primers designed for *VvRRb1* (FJ822984), *VvRRb2*, *VvRRb3* (FJ822985), *VvRRb4* (FJ822986), *VvRRb5* (FJ822987) and *VvRRb6* (FJ822988) we could verify the presence of the predicted fragments with 271 bp, 247 bp, 270 bp, 206 bp, 268 pb and 220 bp respectively (Fig. 27 B).

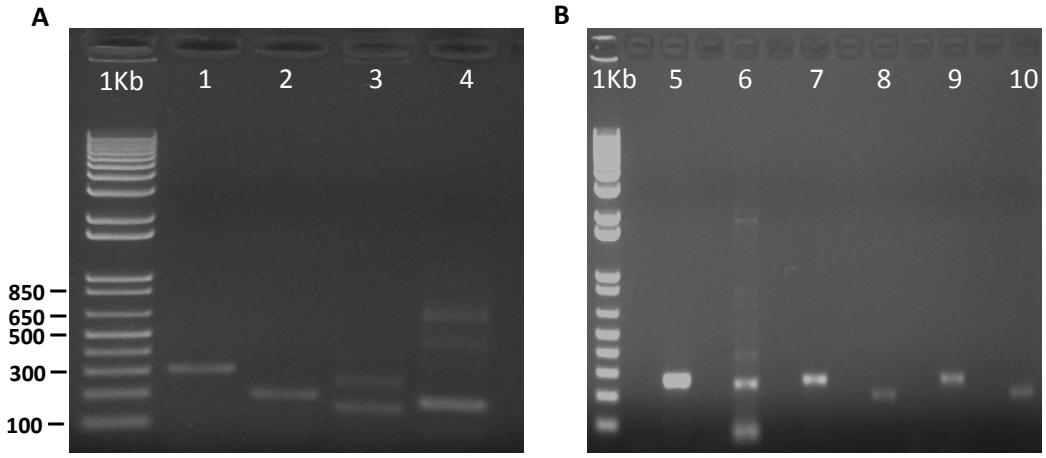

Fig. 27 RR PCR products resolved on 1% (w/v) agarose gels. A) A-type RR and B) B-type RR. 1) *VvRRa1*, 2) *VvRRa2*, 3) *VvRRa3*, 4) *VvRRa4*, 5) *VvRRb1*, 6) *VvRRb2*, 7) *VvRRb3*, 8) *VvRRb4*, 9) *VvRRb5*, and 10) *VvRRb6*.

3.3.2. Cytokinin signalling and meristem identity genes *in TIS plantlets*

Plantlets growing in TIS culture have the advantage of the close similarity to autotrophic plants although growing in controlled, easy to manipulate, *in vitro* conditions.

The analyses of the gels where PCR products were resolved revealed that, as in isolated cells, the primers designed for *VvHP1* never amplified any fragment, neither in root nor in the shoot, reinforcing the idea that this gene is not expressed in *Vitis*. The primers for *VvHP2* and *VvRRb2* (Fig. 28, B, 4, 5, 13) did not amplify any fragment in root samples while all the other primers amplified the shoot and root predicted fragments, with sizes equivalent to cell fragments (Fig. 28 A and B).

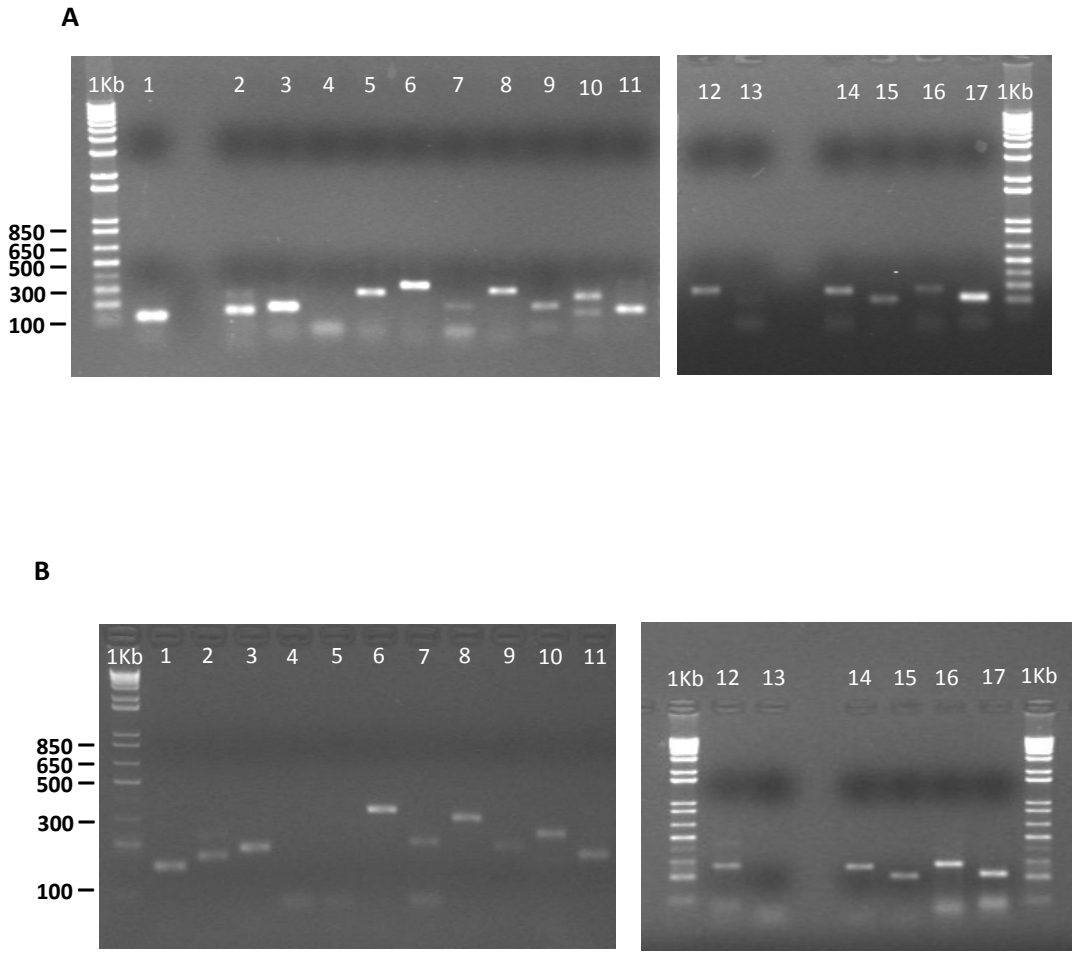

Fig. 28 PCR products resolved on 1.5 % (W/V) agarose gel. A) Shoot and B) Root. 1) *VvCyt1*, 2) *VvCyt2*, 3) *VvCyt3*, 4) *VvHP1*, 5) *VvHP2*, 6) *VvHP3*, 7) *VvHP4*, 8) *VvRRa1*, 9) *VvRRa2*, 10) *VvRRa3*, 11) *VvRRa4*, 12) *VvRRb1*, 13) *VvRRb2*, 14) *VvRRb3*, 15) *VvRRb4*, 16) *VvRRb5*, 17) *VvRRb6*.

The branching results in response to S depletion prompted for the analysis of genes associated to apical and axillary meristem cell identity. For that purpose and using the genes annotated in *Arabidopsis* and deposited at NCBI Database, *WUS*, *CLV3* and *STM* as genes regulated in apical meristem cells and *BRC1*, *BRC2*, *LAS*, *RAX* and *REV* regulated in axillary meristem, as initial queries, we performed a database search and identified *Vitis* homologous, respectively *VvWUS* (FJ822989), *VvCLV3* and *VvSTM,* and genes *VvBRC1*, *VvBRC2*, *VvLAS* (FJ822990), *VvRAX* and *VvREV*.

In the analyses of PCR products with primers designed for *VvWUS* no fragments were amplified in roots and only a slight presence was observed in shoots. The other two apical meristem genes were present in gels prepared with root and shoot cDNA. These products have the predicted sizes of 135 bp, 106 bp and 102 pb respectively for *VvWUS*, *VvCLV* and *VvSTM* (Fig. 29 A). All axillary meristem genes were present in the analyzed tissues (Fig. 29 B). The PCR fragments from shoot tissue amplified with primers for *VvBRC1*, *VvBRC2*, *VvLAS*, *VvRAX* and *VvREV* showed the expected sizes of 176 bp, 106 bp, 120 bp, 117 bp and 109 bp, respectively.

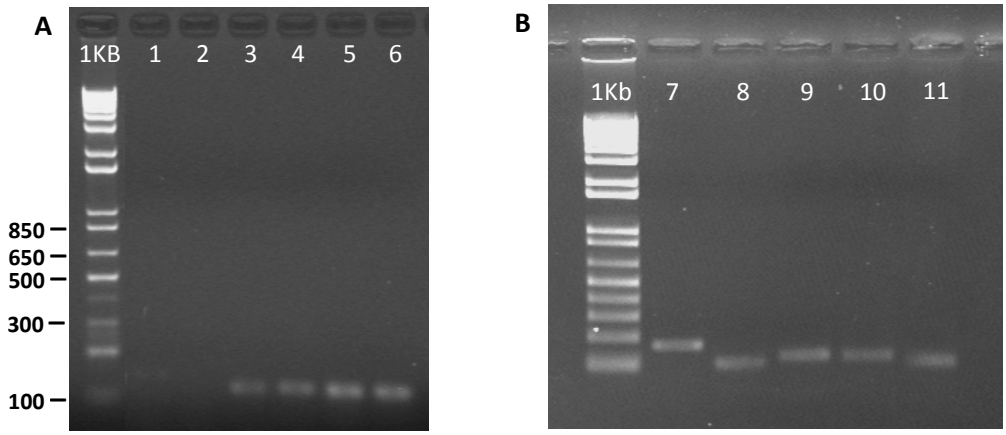

Fig. 29 Apical meristem and axillary meristem PCR products resolved in 1 % (w/v) agarose gels in Shoot (S) and root (R). A) apical meristem genes, B) axillary meristem genes. 1) *VvWUS* S, 2) *VvWUS* R, 3) *VvCLV* S, 4) *VvCLV* R, 5) *VvSTM* S, 6) *VvSTM* R, 7) *VvBRC1* S, 8) *VvBRC2* S, 9) *VvLAS* S, 10) *VvRAX* S, 11) *VvREV* S.

3.4. Expression of cytokinin signalling and apical and axillary meristem genes: effect of –S conditions

Expression levels of transcripts associated with cytokinin signalling pathway and apical and axillary meristem identity were analysed by qrt RT-PCR. Preliminary results from dilution analysis of the whole set of transcripts identified the candidate genes for further analysis. The expression patterns of *VvCyt1, VvCyt2, VvCyt3, VvHP2, VvHP3, VvRRa3*, *VvRRa4*, *VvRRb5*, *VvRRb6*, *VvWUS*, *VvCLV*, *VvSTM*, *VvBRC1*, *VvBRC2*, *VvLAS*, *VvRAX* and *VvREV* were then quantified by qrt RT-PCR.

3.4.1. Vitis cells in the presence of cytokinin

In cells growing in the presence of cytokinin, *VvCyt1* and *VvCyt3* were not affected by the absence of sulfur. The expression levels of *VvCyt1* and *VvCyt3* did not change significantly along the 7 days treatment. As presented in Fig. 30, *VvCyt2* was the only cytokinin receptor gene that responded to sulfur deficiency with a downregulation of 10 times as compared to + S, at day 7.

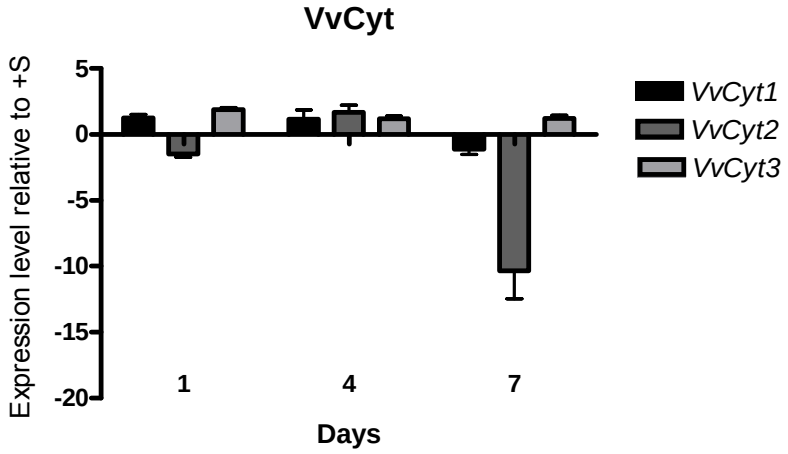

Fig. 30 Expression levels of VvCyts genes as quantified by real time PCR at days 1, 4 and 7 of -S treatment in the presence of cytokinin.

Concerning the expression of HPs genes, *VvHP2* seemed to be unaffected by the –S during the 7 days of treatment. Only *VvHP3*, showed an 18 times decrease of its expression at day 7 (Fig. 31).

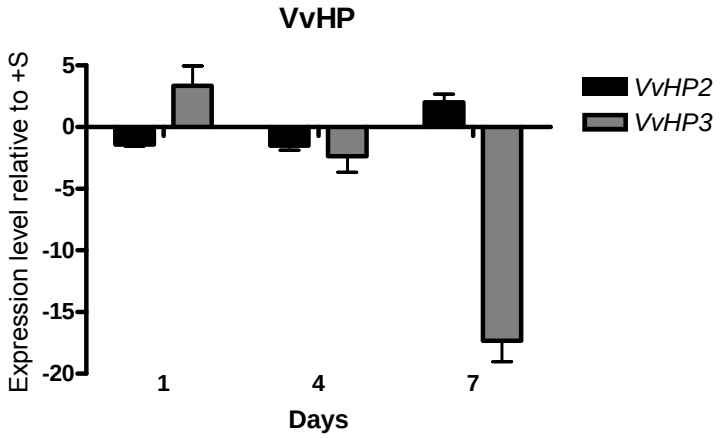

Fig. 31 Expression levels of VvHPs genes as quantified by real time PCR at days 1, 4 and 7 of -S treatment in the presence of cytokinin.

The analysis of VvRRs genes expression showed that B-type *VvRR*s were unaffected by sulfur depletion. The first 4 days of treatment didn't show any response in the expression of tested genes. Only exception is *VvRRa4*, whose expression was reduced of about 7 times, at day 7 (Fig. 32).

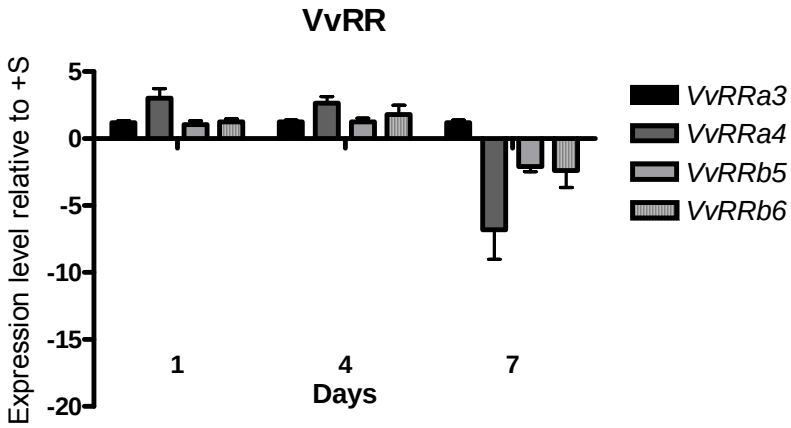

Fig. 32 Expression levels of VvRRs genes as quantified by real time PCR at days 1, 4 and 7 of -S treatment in the presence of cytokinin.

The meristem genes *VvWUS*, *VvClv* and *VvSTM* are responsible for apical meristem identity and respond to hormonal regulation during meristem cell division. In the present cell system, although there is the presence of cytokinin, these genes were not affected by sulfur starvation (Fig. 33), during the 7 day treatment.

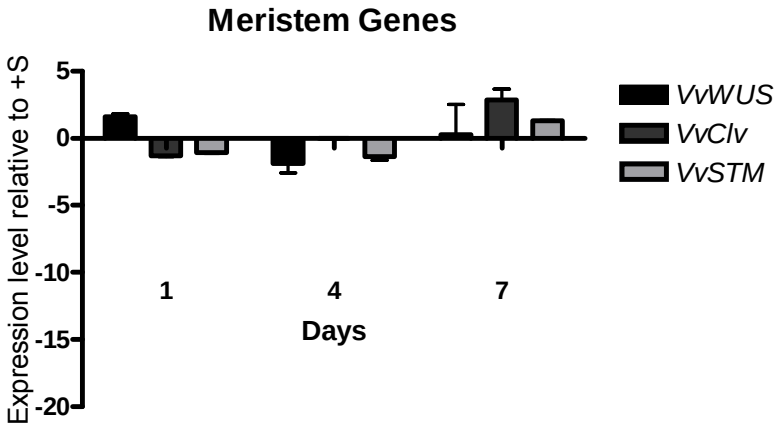

Fig. 33 Expression levels of *Vitis* apical meristem genes as quantified by real time PCR at days 1, 4 and 7 of -S treatment in the presence of cytokinin.

3.4.2. Vitis cells in the absence of cytokinin

It is well documented that cytokinin signal is regulated by components of its own signal pathway. To evaluate if the absence of cytokinin had any influence in the expression of the studied genes, a parallel experiment was conducted maintaining S treatment but removing the hormone from the medium.

As shown in Fig. 34, the general response of the expression of cytokinin receptors was similar to that verified in the presence of the hormone. However, at day 7 the three receptor genes showed a tendency for downregulation with *VvCyt1* showing a downregulation of 7 times in –S as compared to the control +S, when in the presence of cytokinin that downregulation attained the expression of *VvCyt2* (compare Fig. 34 to Fig. 30).

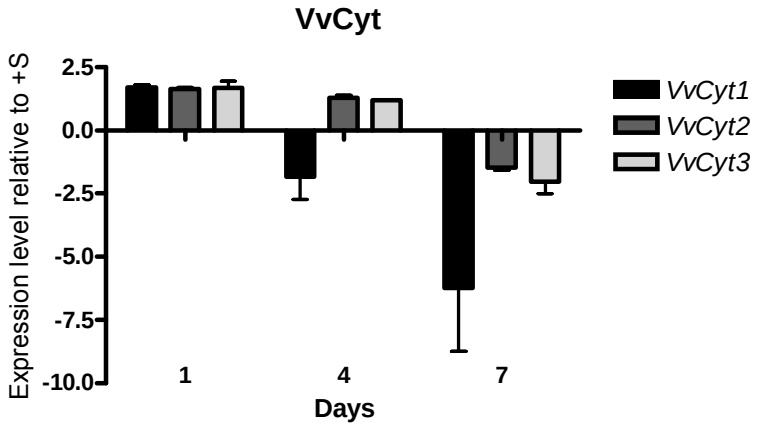

Fig. 34 Expression levels of VvCyts genes as quantified by real time PCR at days 1, 4 and 7 of -S treatment in the absence of cytokinin.

Considering the expression of HPs genes at day 7, *VvHP2* had an alteration of its expression by downregulation of 5 times in –S when, in the presence of cytokinin, the downregulation was verified for *VvHP3* (compare Fig. 35 to Fig. 31).

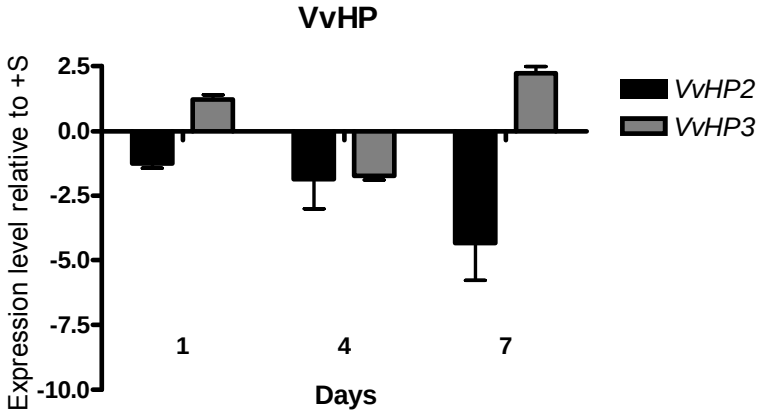

Fig. 35 Expression levels of VvHPs genes as quantified by real time PCR at days 1, 4 and 7 of -S treatment in the absence of cytokinin.

The analysis of VvRRs genes, showed that only *VvRRa3* was affected by sulfur depletion in the absence of cytokinin, with an up-regulation of five fold in day one and more than 10 times in day 7, a response significantly different of that obtained in the presence of cytokinin where the expression of this transcript was unaffected by the S starvation (compare Fig. 36 to Fig. 32).

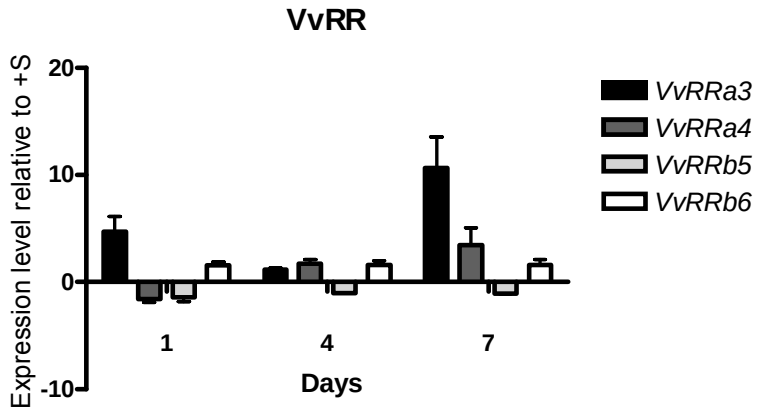

Fig. 36 Expression levels of VvRRs genes as quantified by real time PCR at days 1, 4 and 7 of -S treatment in the absence of cytokinin.

The apical meristem genes (*VvWUS*, *VvClv* and *VvSTM*) in cells growing in the medium without cytokinin have all the same behaviour as in the presence of the hormone. The expression of these transcripts was detected but they were not affected by sulfur or cytokinin starvation during the 7 days of treatment (compare Fig. 37 to Fig. 33).

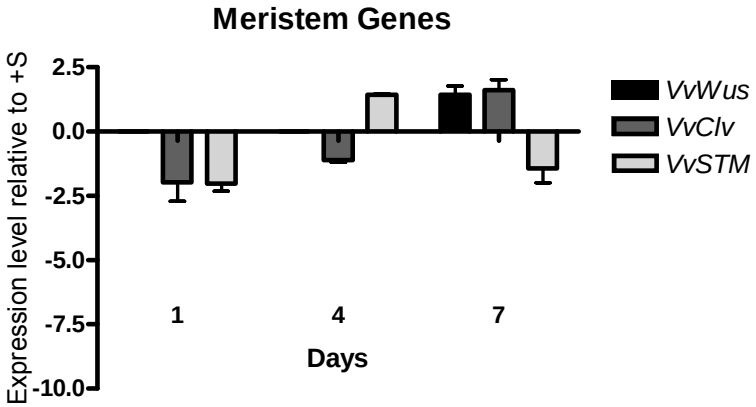

Fig. 37 Expression levels of *Vitis* apical meristem genes as quantified by real time PCR at days 1, 4 and 7 of -S treatment in the absence of cytokinin.

3.4.3. Vitis GM shoots

In full heterotrophic conditions as it occurs in GM medium, *VvCyt* genes were unaffected by the absence of sulfur since the expression levels of all *VvCyt* were not significantly different after 4 weeks treatment (Fig. 38).

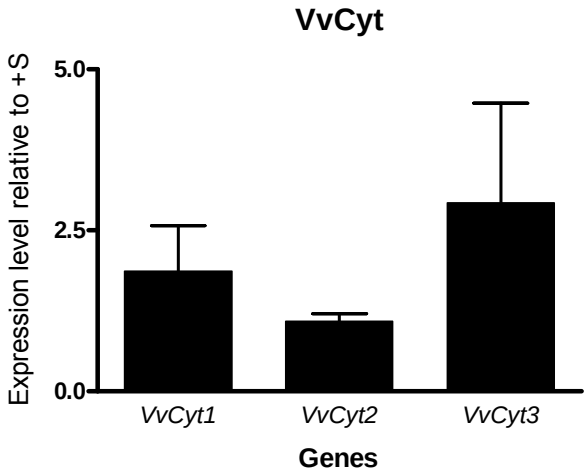

Fig. 38 Expression levels of VvCyt genes as quantified by real time PCR in GM after 4 weeks -S treatment.

In the cytokinin signalling pathway HP genes come downstream of the cytokinin receptors. A slight downregulation of three times during the 4 weeks in –S medium was measured for *VvHP2* (Fig. 39).

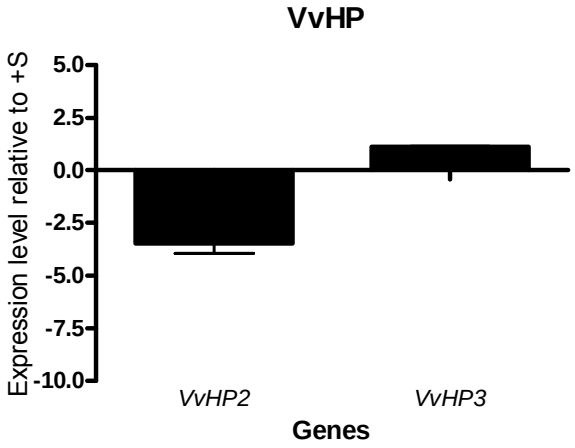

Fig. 39 Expression levels of VvHP genes as quantified by real time PCR in GM after 4 weeks -S treatment.

A-type RR are known as cytokinin primary-response genes. The analysis of *VvRR* genes expression revealed that only A-type response regulators responded to –S condition. As in cells in the absence of cytokinin *VvRRa3* was the only transcript to respond to -S conditions but, while an up-regulation was measured in isolated cells (Fig. 36), here its transcription level was reduced by more than three fold (Fig. 40).

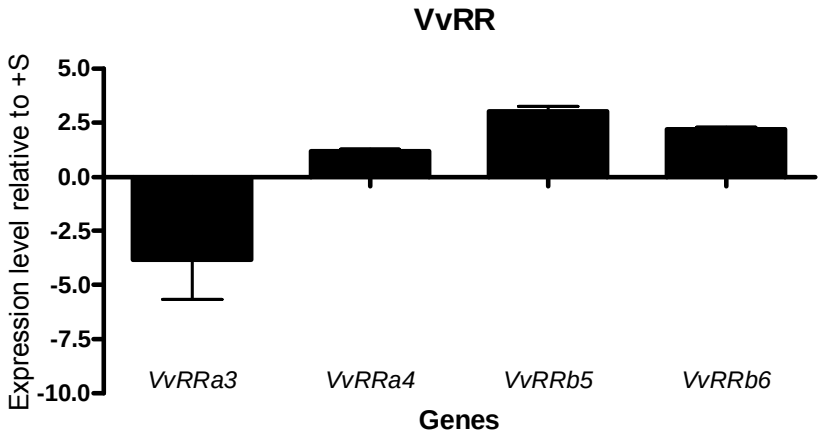

Fig. 40 Expression levels of VvRRs genes as quantified by real time PCR in GM after 4 weeks -S treatment.

The transcripts of all apical meristem genes were expressed in shoots, with *VvSTM* being affected by sulfur starvation by a downregulation of about 8 times (Fig. 41). The transcription level of the other apical meristem genes (*VvWUS* and *VvClv*) are not affected by the absence of sulfur.

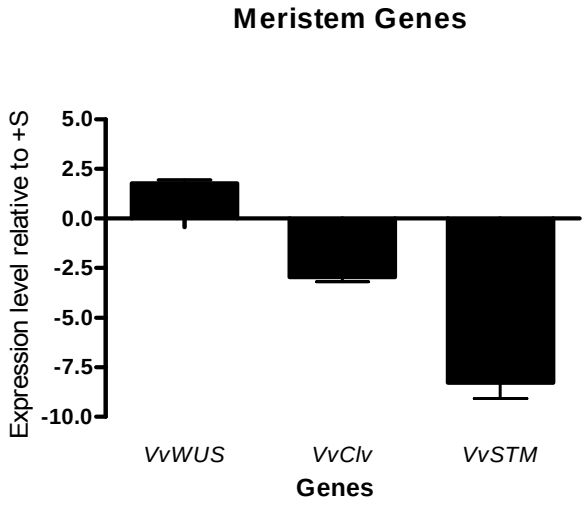

Fig. 41 Expression levels of *Vitis* apical meristem genes as quantified by real time PCR in GM after 4 weeks -S treatment.

In the analysis of axillary meristem genes expression only *VvRAX* and *VvREV* were not affected by –S condition. The other genes, *VvBRC1*, *VvBRC2* and *VvLAS* were downregulated in their expression level by 8 and 11 times, respectively, for the former while *VvLAS* had a severe downregulation of more than 40 times (Fig. 42).

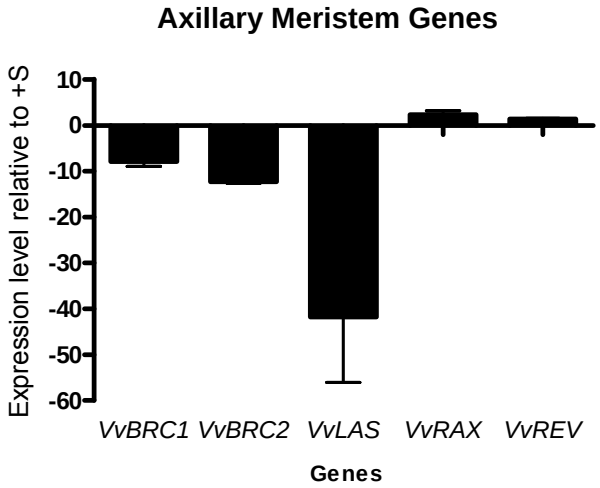

Fig. 42 Expression levels of *Vitis* axillary meristem genes as quantified by real time PCR in GM after 4 weeks -S treatment.

3.4.4. Vitis TIS plantlets

As referred previously, the tissue culture system TIS combines sterile heterotrophic conditions in liquid temporary immersion with the positive effects of renewable aeration. Altogether, this *micropropagation* technique occurs in conditions similar to autotrophic environment.

Concerning the expression of *VvCyt*s genes in plantlet shoots under +S and –S nutrition, only *VvCyt2* was upregulated, by *ca* 7 times. Root *VvCyt* genes seem not be affected by sulfur starvation, with low and equivalent expression levels (Fig. 43).

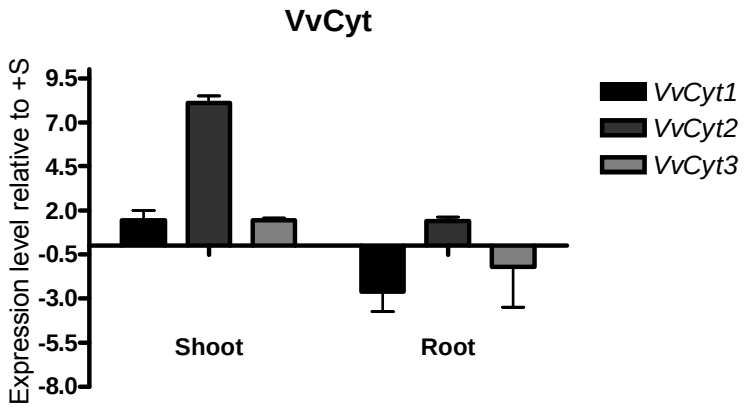

Fig. 43 Expression levels of VvCyt genes in TIS plantlets under +S and of -S treatment for two weeks as quantified by real time PCR.

The expression of HPs genes as shown in Fig. 43, *VvHP2* and *VvHP3*, in the shoot, seemed to be unaffected by -S treatment, while in root *VvHP2* had an upregulation of 8 times relative to +S (Fig. 44).

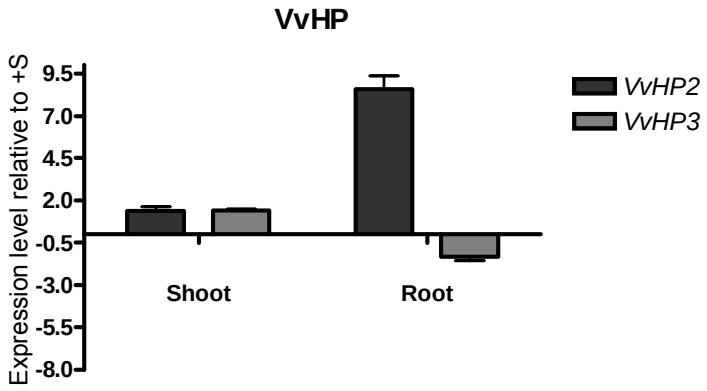

Fig. 44 Expression levels of VvHP genes in TIS plantlets under +S and of -S treatment for two weeks as quantified by real time PCR.

Concerning the level of expression of *VvRR*s genes revealed that A-type response regulators do not respond to sulfur starvation either in shoots or in roots (Fig. 45). *VvRRb*s showed a tendency for a slight down-regulation of *ca* 4 times in the shoot and in the root, but only for *VvRRb6*, with an expression decrease of more than 3 fold (Fig. 45).

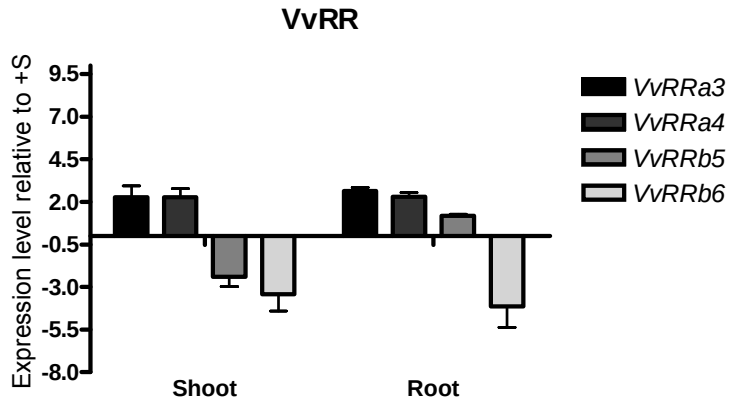

Fig. 45 Expression levels of VvRR genes in TIS plantlets under +S and of -S treatment for two weeks as quantified by real time PCR.

The transcripts of all apical meristem genes were detected in the plantlet shoot. In the root *VvWUS* transcripts were absent. None of the expressed genes were responsive to sulfur starvation (Fig. 46).

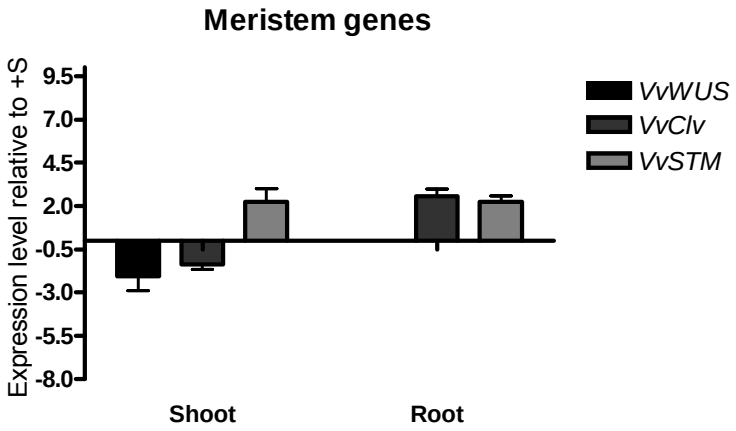

Fig. 46 Expression levels of *Vitis* apical meristem genes in TIS plantlets under +S and of -S treatment for two weeks as quantified by real time PCR.

The transcripts of all axillary meristem genes were detected in the shoot and root of TIS plantlets. As the apical meristem genes, the transcription rates of axillary genes (*VvBRC1*, *VvBRC2*, *VvLAS*, *VvRAX* and *VvREV*) were not affected by sulfur starvation (Fig. 47).

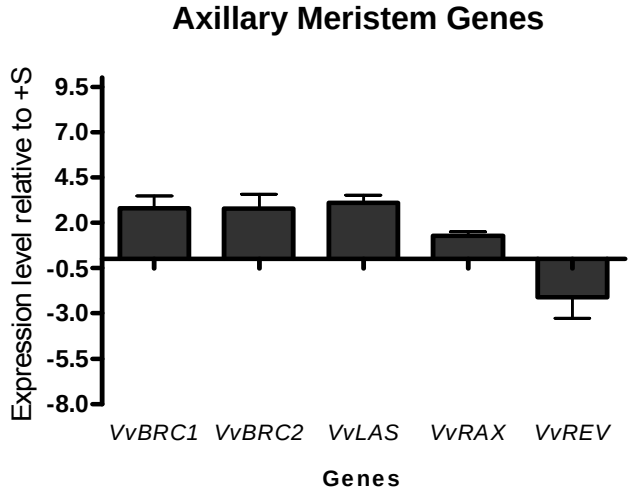

Fig. 47 Expression levels of *Vitis* axillary meristem genes in TIS plantlets under +S and of -S treatment for two weeks as quantified by real time PCR.

4. Discussion

Due to its involvement in essential metabolic pathways, sulfur has a marked effect on plant growth and productivity. Sulfur used by crop plants resulted from sulfur-containing fertilizers and/or sulfur in rainfall (Jolivet, 1993). Due to environmental policies both these sources were significantly reduced. Recent studies indicate that sulfur deficiency can be a limiting factor to crop yield and quality (Saito, 2004; Hawkesford, 2005). Therefore, former research on excessive sulfur due to atmospheric pollution moved to the effects of S-deficiencies. The beneficial effects of sulfur as a potent agent against grapevine pathogens are reported since ancient times (Rausch, 2007).

Cytokinins are essential plant hormones that control cell division, shoot meristem initiation, leaf and root differentiation, chloroplast biogenesis, stress tolerance and senescence (Müller and Sheen, 2007). Cytokinin was found to be a negative regulator of sulfur acquisition (Maruyama-Nakashita *et al.*, 2004) by down-regulating the expression of sulfate transporter genes and the sulfate uptake, both induced by sulphur limitation. Maruyama-Nakashita *et al.* (2004) suggest those two independent modes of regulation for sulfate acquisition, one dependent on sulfate depletion and one dependent on cytokinin. The evidence for the involvement of cytokinin signalling –S response came from the genetic study of the cytokinin receptor mutant CRE1 (Maruyama-Nakashita *et al*., 2004). Our main aim was to assess the influence of sulfur starvation on *Vitis* cytokinin signalling pathway and in meristem identity genes.

Plant model systems analysed in controlled experimental conditions are useful tools to assess limitant nutrient situations as it is the case for sulfate deficiency. In a previous study with *Vitis* cell cultures sufate proved to be essential for normal growth (Tavares *et al*., 2008). Here we show that in *callus*, a second *Vitis* model system applied in the present study, fresh weight was significantly affected by sulfur deficiency. Although *callus* fresh weight in the absence of sulfate was not significantly different from full sulfate conditions in the first two weeks certainly due to previous sulfate accumulation, in the fourth and sixth week in –S the *callus* fresh weight reduced drastically comparing to the control. These results are corroborated by RGR data, which were negative in *callus* in –S medium from the fourth week ahead. In order to analyse a differentiated system under manipulated nutrient conditions, *in vitro* cultures of grapevine shoots in gel medium (GM) were used. *In vitro* shoots in -S maintained the initial fresh weight and RGR not far from null, confirming the need of sulfate for normal growth. As expected, the –S conditions affected significantly the total chlorophyll

content, confirming the impairment of basic physiological mechanisms by the lack of a macronutrient as sulfur.

Most of the sulfate, as well as nitrate, taken up by plants is used in protein synthesis. In plant cell cultures –S condition often results in the accumulation of free amino acids in parallel with a decrease in protein content (Amâncio *et al.*, 1997). Furthermore, when external S concentrations are low, plants do not redistribute S compounds to growing tissues, with an immediate response at growth and chlorophyll content levels (Cram, 1990).

Temporary Immersion System (TIS) has proven to be an alternative to gel medium (GM) as an efficient propagation technique in what concerns plantlet morphology and physiology (Lorenzo *et al.,* 2001; Escalona *et al.,* 2003; Aragón *et al.,* 2005). Multiplication of *Vitis* shoots in –S medium confirmed the use of this culture method since –S reduced to ca 50% the multiplication rate, a suitable parameter for validating the hypothesis that the effect of S depletion can operate through cytokinin signalling.

Multiplication by tissue culture techniques grounds on the reversion of apical dominance by cytokinins. The branch impairment measured as a reduction in the number of new shoots in the shoot system used in the present study was only clearly verified from the third week on; the absence of effect during the first two weeks is certainly due to previous sulfate accumulation. These results can indicate that cytokinin signal pathway or the genes necessary for apical or axillary meristem identity are affected by sulfate starvation.

Then our first approach was to test the effect of S depletion on cytokinin transduction signalling genes.

It has been demonstrated that cytokinin is sensed by membrane-located HK receptors that transmit the signal by a multi-step phosphorelay (HPs) to the nucleus RRs that activate or repress transcription (Kiba *et al.*, 2003). The signal is perceived and transmitted by a phosphorelay system through a complex form of the two-component signalling pathway which has long been known in prokaryotes and lower eukaryotes (Hwang and Sheen, 2001). Recent studies have described the major components of the cytokinin signalling pathway in *Arabidopsis* (Hwang and Sheen, 2001) maize (Asakura *et al.*, 2003) and rice (Ito and Kurata, 2006). Whereas *Vitis* genome was recently published (Jaillon *et al.*, 2007; Velasco *et al.*, 2007) it was possible to analyse for the first time the pathway in this species, providing new insights into *Vitis* cytokinin signalling.

From database searches we identified three (HKs) cytokinin receptors, three phosphotransfer proteins (HPs), 4 A-type RRs and 6 B-type RRs. The three *Vitis* cytokinin receptors *VvCyt1*, *VvCyt2* and *VvCyt3* maintain a conserved CHASE domain, a His Kinase A domain and a Signal receiver domain, confirming them as cytokinin Histidine Kinases receptors. The phylogenetic analysis of *Vitis* receptors showed variable but high degree of homology (65 %) between *Arabidopsis* and *Vitis*. The degree of homology (58 %) to genes of the monocots maize and rice point to ancestral evolution of the pathway (Müller and Sheen, 2007).

Downstream of the cytokinin receptor are the HP genes. From the 4 HP genes present in *Arabidopsis* only *VvHP1* was not expressed in any *Vitis* tissues analyzed so far. The three HPt genes (*VvHP2*, *VvHP3* and *VvHP4*) identified in *Vitis* genome encode a Histidine Phosphotransfer domain common to all HPts. The phylogenetic analysis of *Vitis* HPs genes clustered into two groups, one formed by sequences homologous to *Arabidopsis*, and the second group enclosing sequences similar to rice, confirming the conservation of the pathway during evolution process (Müller and Sheen, 2007).

The phosphate group present in HPs is then transferred to RRs (Hwang and Sheen, 2001). The RRs are categorized into two groups. Ten A-type response regulators genes were annotated in *Arabidopsis* genome while in *Vitis* only 4 genes were identified so far. The presence of A-type RRs in other species, *e.g.* rice, and the degree of homology of *Vitis* genes suggests they may play a similar role to A-type ARRs.

The B-type RRs form the other RR group. These genes mediate the cytokinin signal to A-type RR. Comparing to ten B-type ARRs we have identified 6 *Vitis* B-type response regulators. The degree of homology of *Vitis* genes also suggests that they may play a similar role to B-type ARRs.

These results confirm that the “two-component system” predicted for cytokinin signalling pathway in *Arabidopsis* and rice also applies to *Vitis*. According to Müller and Sheen (2007) cytokinin signalling pathway is conserved in all plant species, suggesting an evolutionary conservation of this pathway.

Real-time PCR is increasingly used in plants to study the expression patterns of particular genes in different experimental conditions, allowing the detection of a given target gene in a rapid, specific and sensitive manner (Gachon *et al.*, 2004). Therefore to ascertain the effects

of –S conditions, real-time PCR was elected to quantify the expression of key genes of *Vitis* cytokinin signalling pathway.

Explants of the *cre1-1* mutant, which has a point mutation in the Histidine Kinase domain, showed a lack of typical cytokinin responses such as cell proliferation and subsequent shoot formation (Hwang and Sakakibara, 2006). The downregulation of *VvCyt1* and *VvCyt2* in sulfur deficient isolated cells irrespectively to the presence of cytokinin suggests that cytokinin receptors are not induced by the availability of the hormone. The lack of response to sulfur depletion of cytokinin receptor genes in *Vitis* shoots steers the different response of these genes to sulfur in dedifferentiated and differentiated cells.

AHPs play a major role in cytokinin signalling, and the quintuple *Arabidopsis ahp* mutant (*ahp1,2,3,4,5*) has various abnormalities in growth and development, including reduced fertility, increased seed size, reduced vascular development, and a shortened primary root (Hutchison *et al.*, 2006). The expression of HPs genes (*VvHP2* and *VvHP3*) in isolated cells and shoots followed an equivalent pattern to cytokinin receptor genes, confirming the different regulation occurring in isolated cells and differentiated tissues.

Regarding *VvRRa3* and *VvRRa4* in –S isolated cells, the presence or absence of cytokinin affected their expression differently, since *VvRRa4* was downregulated in the presence of the hormone but *VvRRa3* was upregulated after cytokinin removal. Hwang and Sheen (2001) propose A-type ARRs as negative regulators of cytokinin signalling because their disruption has a stimulatory effect on cytokinin signalling. Conversely, in experiments carried out by Brenner *et al.* (2005) in *Arabidopsis*, A-type RRs are upregulated when cytokinin is applied to the plants. How can we reconcile these findings? A possibility is related with the bell shape of cytokinin response curve, with a well defined optimum concentration (Taiz and Zeiger, 2006). The authors show that root supraotimal levels of endogenous cytokinin inhibit growth which is resumed when endogenous cytokinin levels are lowered by overexpressing cytokinin oxidase, while further decrease in the cytokinin content restores the inhibition of root growth. An equivalent rationale can be applied to *Vitis* cells. The fresh weigh of *Vitis* cells without cytokinin is significantly affected in 7^{th} day. This suggests a scenario in which a stepwise activation of different cytokinin pathway is initiated by upregulation or downregulation of transcriptional control factors (Brenner *et al.*, 2005). Osakabe *et al.* (2002) showed that overexpression of *ARR4* in transgenic cultured stems markedly promoted shoot formation in the presence of cytokinin, while overexpression of *ARR8* repressed greening and shoot formation of *calli*. These data suggest that different experimental systems have different behaviours, depending on their degree of complexity. Conversely to a more complex system

like shoots unaffected by sulfur starvation, in *Vitis* cells sulfur depletion seems to interrupt the cytokinin signal pathway at RRa level.

From *Arabidopsis* results, the B-type RR genes are apparently not induced by cytokinin but can be involved in the transcription of cytokinin primary target genes (Kiba *et al.*, 1999; D'Agostino *et al.*, 2000 and Hwang and Sheen, 2001). The behaviour of *Vitis* B-type RRs was equivalent, since they did not respond either to cytokinin or S starvation. Only in TIS the B-type RRs were downregulated, suggesting a different regulation of cytokinin signalling.

As a whole, in *Vitis* cells different cytokinin signalling genes, namely *VvCyt*s, *VvHP*s and A-type *RR* were downregulated by sulfur starvation independently of cytokinin. The unique up-regulation of A-type RR in the absence of cytokinin suggests A-type RR as the cytokinin primary-response (D'Agostino *et al.* 2000) as negative regulator genes (Hwang and Sheen, 2001).

TIS conditions approach the environment of autotrophic growth. This feature can explain the different results obtained in response to –S treatment as compared to GM explants. The less notorious branching inhibition verified in TIS –S plantlets can be associated to the downregulation of B-type *VvRR*s. Hypocotyl elongation assays in B-type RRs knock-out *Arabidopsis* showed that the sensitivity to cytokinin was affected, reducing the hypocotyls elongation (Hass *et al.*, 2004). B-type RRs act as transcription factors for A-type RRs transcription (Lorsmann *et al.*, 2001), then a downregulation of B-type RRs must imply that A-type RR are not transcribed, impairing the downregulation of cytokinin signal verified in GM conditions.

The plant shoot is derived from the primary shoot apical meristem (SAM) whose activity is regulated by environmental inputs, such as nutrient availability, that can be relayed by plant hormones (Ongaro and Leyser, 2007). We identified three apical meristem genes *VvWus*, *VvClv* and *VvSTM* and analysed their expression in cell cultures and shoots. The three genes were transcribed in *Vitis* cells but were unaffected by sulfur depletion, eventually because cells correspond to a dedifferentiated system. In shoots and plantlets the three transcripts are expressed, but *VvWus* is absent in the root. Only *VvSTM* expression decreased in –S GM shoots. The different roles of cells within the meristem are controlled by different regulatory genes. One of the earliest genes expressed is *WUS* (Bowman and Eshed, 2000) that encodes a homeodomain protein (Lindsay *et al.*, 2006). *WUS* is required for stem cell identity (Lenhard *et al.*, 2002), conferring stem-cell identity to overlaying

neighbouring cells (Mayer *et al.*, 1998). In *Arabidopsis CLV1* (*CLAVATA1*) and *CLV3* (*CLAVATA3*) are important genes for meristem maintenance. CLV1 is a leucine-rich repeat (LRR) receptor-like kinase (Lindsay *et al.*, 2006) and mutations is this genes lead to an accumulation of cells in the central zone. Mutations in *WUS* and *CLV1/CLV3* have essentially opposite effects on the cell population, suggesting that these genes act in pathways that promote and restrict cell division respectively (Bowman and Eshed, 2000). In *Arabidopsis* it is well demonstrated that *Shootmeristemless (STM)*, a Homeobox transcription factor, is required for the establishment or maintenance of stem cell fate (Bowman and Eshed, 2000) which is critical for the initiation of primary shoot apical meristem (Barton and Poethig, 1993). One of the earliest indicators of a switch in fate from indeterminate meristem to determinate leaf primordium is the down-regulation of *KNOX1* genes orthologous to *STM* in the incipient primordial. In *Arabidopsis thaliana*, loss-of-function of the *STM* gene results in loss of the SAM, in agreement with its role in meristem identity acquisition/maintenance (Long *et al.*, 1996). Since *Vitis VvSTM* is downregulated, in –S conditions it is reasonable to assume that apical meristem is not properly formed in sulfur starvation, and this may be one of the reasons of *Vitis* branching impairment.

Shoot branching is the process by which axillary buds, located on the axil of a leaf, develop and form new branches. The process by which a dormant bud activates and becomes an actively growing branch is complex and very finely tuned. Bud outgrowth is regulated by the interaction of environmental signals and endogenous ones, such as plant hormones. Hormones known to have a major influence are auxin, cytokinin, and a novel, as yet chemically undefined, hormone. The novel hormone also moves acropetally as cytokinin but it inhibits bud outgrowth (Ongaro and Leyser, 2007). The identity of axillary meristems (AM) must be equivalent to SAM but differentially regulated (Greb *et al.*, 2003), once the fate of axillary buds is to grow out to give a branch or to remain dormant in the axils of leaves (Aguilar-Martínez *et al.*, 2007).

Shoot multiplication *in vitro* is based on the inhibition of apical dominance and forced branching by adding exogenous cytokinin. *In vitro* shoots might then comprise a suitable model for analysing axillary meristem gene expression. Among the genes responsible for axillary meristem fate are *Arabidopsis* TCP transcription factors *BRANCHED1* (*BRC1*) and *BRANCHED2* (*BRC2*) (Aguilar-Martínez *et al.*, 2007) the transcription factor from GRAS family *LATERAL SUPPRESSOR* (*LAS*) (Greb *et al.*, 2003), the MYB gene *REGULATOR OF AXILLARY MERISTEMS* (*RAX*) (Keller *et al.*, 2006; Müller *et al.*, 2006) and the

Homeodomain-leucine zipper protein that acts like transcription factor *REVOLUTA* (*REV*) (Talbert *et al.*, 1995; Otsuga *et al.*, 2001;).

An *in silico* approach followed by the amplification of the transcripts with specific primers designed for each gene allowed to identify in *Vitis* the homologous *VvBRC1*, *VvBRC2*, *VvLAS*, *VvRAX* and *VvREV* genes. In GM shoots *VvRAX* and *VvREV* seemed to be unaffected by sulfur starvation, while the other genes were downregulation. *BRC1* responds to developmental and environmental stimuli controlling branching and when expressed in developing buds, arrests bud development. Genes promoting bud arrest have been described in monocots, *e.g. teosinte branced1* (*tb1*) from maize (Doebley *et al.*, 1997) and its homologs from rice *Os tb1* (Hu *et al.*, 2003; Takeda *et al.*, 2003). In *Arabidopsis* when *BRC1* and *BRC2* are downregulated the number of rosette branches increase, indicating that *BRC1* and *BRC2* retard all stages of bud development. Mutant and expression analyses suggest that *BRC1* is required for auxin-induced apical dominance. Therefore, *BRC1* acts inside the buds as an integrator of signals controlling bud outgrowth and translates them into a response of cell growth arrest. LAS exerts important roles in very diverse processes such as signal transduction, meristem maintenance and development (Bolle, 2004). *LAS* transcripts accumulate in the axils of all primordial derived from the SAM. In *Arabidopsis*, the *LAS* gene is an essential component of the genetic mechanism governing the acropetal gradient of axillary meristem formation. Comparison of the phenotypes of tomato and Arabidopsis lateral suppressor mutants revealed that the described control mechanism is conserved during evolution (Greb *et al.*, 2003). *RAX* expression is spatially more restricted than *LAS*, restricted to a small central domain within the boundary zone separating SAM and leaf primordia during early leaf primordium development and is currently the earliest spatial marker for future AMs. *RAX* is therefore the earliest known specific marker for AM position (Keller *et al.*, 2006). *REV* genes are necessary to promote the normal growth of apical meristem acting either indirectly to establish meristem identity or directly to activate the expression of other meristem regulators (Otsuga *et al.*, 2001). The *rev-1* mutation caused overgrowth of both rosette and cauline leaves (Talbert *et al.*, 1995). These findings suggest that *REV* is involved in limiting cell division. REV acts either indirectly to establish meristem identity, or directly to activate the expression of other meristem regulators (Otsuga *et al.*, 2001).

LAS gene is necessary for axillary meristem initiation. Mutations in this gene showed a severe reduction in the number of axillary shoots (Greb *et al.*, 2003). The downregulation of *VvLAS* is one of the explanations for the decrease in *Vitis* branching. The apparent contradictory result of expression patterns of *VvBRC1*, *VvBRC2* and *VvLAS* may be solved by the *VvLAS* function upstream of *VvBRC1* and *VvBRC2*, preventing any phenotypic

expression due to the activation of the latter genes. Taken together, the expression patterns of *VvSTM* and *VvLAS* could explain the branching pattern of GM shoots in response to S deficiency.

5. Conclusion

Sulfur is essential macronutrient for plants. *Vitis* shoots growing *in vitro* in full MS medium responded drastically to sulfur starvation. The responses in sulfur uptake and metabolism level namely the reversion of apical dominance inhibition triggered by routinely added cytokinin. These explants developed symptoms of growth impairment and branching inhibition. One possible explanation is the interference of sulfur with cytokinin signal pathway genes or with the expression of transcription factors acting at SAM or AM level. Our aim was to assess whether a -S situation affects *Vitis* physiology and the cross talk between the hormone signalling and sulfur status deserves further investigation at the molecular level.

Sulfur nutrition proved to be essential for grapevine sustained growth as put in evidence by the results of experiments carried out with undifferentiated cells and *callus* and differentiated *in vitro* shoots.

Differentiated *in vitro* model systems where apical dominance is inhibited by exogenous cytokinin addition allowed to identify the drastic effect of S deficiency on shoot branching stimulating to investigate the effect of -S on cytokinin signalling pathway

Thanks to the opportunity brought about by the recent sequencing of *Vitis* genome, it was possible to confirm in *Vitis* the “two-component system” predicted for cytokinin signalling pathway in *Arabidopsis* and rice.

The results present in this work suggest that cytokinin signalling pathway is affected by sulfur starvation, but differently according to the models system.

The different roles of cells within the meristems are controlled by different regulatory genes. To ascertain whether the branching reduction in shoots under –S treatment a line of research focused on the expression of shoot apical meristem (SAM) and axillary meristem (AM) genes. The presence, in *Vitis*, of transcripts of most of those genes annotated in *Arabidopsis* comprises a stimulating result. The long term influence of Sulfur deficiency crosstalk with cytokinin signalling and meristematic activity needs further investigation.

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Supplementary data

Suplementary data I – Culture mediums

Callus and Cells medium

	Concentration
MS	0.2203 g
PVP 40T	0.5 %
Sacarose	2 %
2.4 D	2,5 µM
Cinetina	10^{-6} M
Hydrolysate of caseina	0.01 %
gelrite	0.2 %
pH	5.7

Vitis multiplication mediums

	Fase		
component	**Multiplication**	**Elongation**	**Root expression**
Biotin	0.98 µM	0.98 µM	
Ca Pantothenate	5 µM	5 µM	5 µM
Riboflavin	10 µM	10 µM	
Cistein	133.6 µM	133.6 µM	
NAA	0.50 µM		2 µM
BA	5 µM	1.67 µM	
Sacarose	3 %	2 %	1.5 %
Ascorbic acid	1.76 mg. L^{-1}	1.76 mg. L^{-1}	1.76 mg. L^{-1}
PVP 40T	0.1 %	0.1 %	0.1 %
Gelrite	0.2 %	0.2 %	0.2 %
pH	5.8	5.8	5.8

Altered MS medium for –S

Component	Concentration (mg/100ml)
NH_4NO_3	16,5 g
KNO_3	19 g
$CaCl_2$	3,32 g
$MgCl_2$	3 g
KH_2PO_4	1,7 g
Fe Na EDTA	734 g
$MnCl_2$	1690 g
H_3BO_3	620 g
$ZnCl_2$	860 g
KI	83 g
Na_2MoO_4	25 g
$CoCl_2$	2,5 g
$CuCl_2$	2,5 g
Myo-inositol	2000 g
Nicotinic Ac.	100 g
Piridoxine	100 g
Tiamine	100 g
Glicine	200 g

Suplementary data II – Nucleotide sequence

VvCyt1 (FJ822975)
tgttttaggtatgctgaaaatgctgatggactcgggtcttgatgcaaaccaacaggattatgctgagactgctcatgctagtgggaaagatctaata tcactgataaatgaggttcttgatcaggctaagatagaatcaggaaggctcgagcttgaagctgttccttttgatttgcgtgctgctcttgataatgt tttatcactcttctcaggcaaatctcatgaaaaggggatcgagttggctgtctacatctctgatcaagtgcctgaatttgtcattggagacccygggc gcttcaggcagataattaccaatcttgttggaaattcaataaagttcacacatgacaaagggcatatctttgtctcggtgcatctggcagatgaagt ggtggggccacctgatctcagggacgaagtgctgagacagagcttgaatatagtccatgacagctcaaacaattcctataatacattgagtgggtt tcctgtggtcaacagatggaaaagctgggagaagtttaaaaagttaagctgcacagattcaatggaggaaaccagcataattaaattattagtga ctgtcgaggatacgggtgtgggaataccttcagaagctcaaagtcgaattttcatgccttttatgcaggctgacagttccacttctcgaacgtatggt gggactggaataggattaagcattagcaa

VvCyt3 (FJ822976)
gctgatgtcgtctgtgcagacagtgggaaaagtgcaatcccactgcttaaacctccccacgactttgatgcctgtttcatggatatccagatgccag aaatggaccgggttgaag

VvHP2 (FJ822977)
ctggaggaactgcaggatgatgctaaccctaattttgtagaagaagttgttacattgttttaccgggattcagctcgactcgtccttaacatagacc aggcactggacaagacccctcttgatttttctaagttggacagctacatgcaccagttcaaaggaagtgcttcaagcattggagccaaaaaggta aagggtgaatgcacactgtttagagaatattgcaaggcaggaa

VvHP3 (FJ822978)
gagagcaaccctgattttgtggttgaagtggtgtccctcttctttgaggattctgagaagcttctcaatgatctgtccagagccctagatcagcaa aatgtagatttcaagagggttgattcccatgttcatcagttgaagggcagcagctccagcataggagcacagagagtcaaaaatgcctgcattg cctttcgcaactactgtgaggaacagaacactgatgcgtgcctgagctgcctgcagcaagtgaaacaagagtactcccttgcgaagagcaagc ttgaac

VvHP4 (FJ822979)
cgttaaaatgggaatccatctgaatcagttcatgggaagcagttctagcataggtgctaaaagagtcagaaatgtatgcgttgcttttcgcgcgg cttccgagcagaacaac

VvRRa1 (FJ822980)
atgaattgctcaagaagatcaaggaatcgtccactttccgggaaactccggtggtaatcatgtcatcagaaaacatcctaacgcgaatagaca gatgtttggaggaaggtgcagaggatttcatagtaaagcccgtaaaattgtcggacgtgaagcggctgaaaagattacatgaccagagaaag acagagatggcagtgagggcagagggcatcaataaaagaaagctggga

VvRRa2 (FJ822981)
aaatattggaagcagattccccgcacatttctggttatgggttccgccgccggcgaatttcttcggcatgtgttaccggagaaagttggggtttct gatcactccgctgctgggtcggaggagctccatgttcttgctgttgatg

VvRRa3 (FJ822982)
tgtcatcagaaaacatcccaactcgaatcaataagtgcctggaggaaggagctcaaatgttcatgctaaagcccctcaagcagtctgatgtgaa gaaactaag

VvRRa4 (FJ822983)
aagatcaaggaatcttcatctctgaagacattaccaagttgtgatcagtcctctgagaatatcccctcaaggattaacagatgtctggaagaag gagcagaagatttcttcctgaagccggtcctctatcaggatgtggaa

VvRRb1 (FJ822984)
ctatagcaacaattcaaacagcctccaaatcaagcatcctgagctgtatgttctgctagatgatgactttagccatgcactgctgtctgctccaca acatcttctccaagttgatctccaatgttctgctaatgctgtgtggtccgggacttcagttccagaaagagataagcccggctctgtcatgatcat acctctgtgctcgcaatccagaagtga

VvRRb3 (FJ822985)
tatttgttgaaaccggttcgaattgaggagctcaagaacatatggcaacatgtaatcaggagaaagaagatcgactccaaggaccaaaacaa gtctgnagatcaggacaacgccctacatgcagatggagaaggtggagaaggcccccccatcatccagcaatgcagatcagaatgggaaacta aatagaaagcggaaggaccaaaatgaggatgaggaggaagagggta

VvRRb4 (FJ822986)
aaggggccgncgcctatcacatgctgccatcttttctgaaagccaaggctatggttcaagaaccccggatagcagatgggaagtaaatggtga tactaaagctgagaagccttatacacctagctcttcc cttatgtatg caaagcccct cctgcaaacc tgtggagaaa

VvRRb5 (FJ822987)
cgaccccacttggttgaaaatccttgaaaagatgctcaagaagtgcttgtatgaagtgaccatttgtggtttggcaagggatgctttgaacttgct tcgggaaagaaaagatggatatgacattgtaatcagtgatgttaacatgcctgacatggatggttttaaacttcttgagcttgttgggcttgagat ggatcttccagtaattatgatgtctgttgacggcgaaacaaagcagggtaatga

VvRRb6 (FJ822988)
cttgtctcatgatcttggagaagatgctccggacttgcctttatgaagttactaaatgcaatcgagcagaaacagcattatccctgttgcgaggg aataaaagcgggtttgatattgttataagcgatgtacacatgcccgacatggacggattcaaactccttgagcacattgggc

VvWUS (FJ822989)
ctacacaatggagttaggtccccaagtgctgaacagattcagaggatctcagctaggctgaggcagtacggcaagatcgaaggcaagaaaag ttatct

VvLAS (FJ822990)
gtcgatcctgnggcgtttcattccacgtatctctccctgaaccaaataaccccattcatcaggt

Suplementary data III – Statistics

Fig. 9 statistics

Bonferroni test; variable Var2 (Spreadsheet13)
Homogenous Groups, alpha = ,05000 Error: Between MS = 1,9104, df = 24,000

	Var1	Var2	1	2	3
4	-S6	3,81833	****		
3	-S4	5,20750	****	****	
1	-S0	6,47667	****	****	
5	+S0	7,49333		****	
8	+S6	11,97333			****
7	+S4	12,09000			****
2	-S2	13,75333			****
6	+S2	14,69000			****

Fig. 10 statists

Bonferroni test; variable Var2 (Spreadsheet15)
Homogenous Groups, alpha = ,05000 Error: Between MS = ,06144, df = 16,000

	Var1	Var2	1	2
2	-S4	-0,140588	****	
3	-S6	-0,025595	****	
4	+S2	0,962638		****
1	-S2	1,125815		****
5	+S4	1,165068		****
6	+S6	1,373448		****

Fig.16 statistics

Bonferroni test; variable Var2 (Spreadsheet1)
Homogenous Groups, alpha = .05000 Error: Between MS = 1.4025, df = 66.000

	Var1	Var2 - Mean	1	2	3
4	-S0	1.000000	****		
1	+S0	1.000000	****		
5	-S2	1.833333	****	****	
6	-S3	3.000000		****	
2	+S2	3.000000		****	
3	+S3	5.533333			****

Fig.18 Statistics

Bonferroni test; variable Var2 (Spreadsheet4) Homogenous Groups, alpha = .05000 Error: Between MS = .00000, df = 6.0000

	Var1	Var2 - Mean	1	2	3	4
6	-S3	0.000853			****	
5	-S2	0.001493	****			
4	-S0	0.001787	****	****		
1	+S0	0.001787	****	****		
2	+S2	0.001974		****		
3	+S3	0.002471				****

Fig.19 Statistics

Bonferroni test; variable Var2 (Spreadsheet6) Homogenous Groups, alpha = .05000 Error: Between MS = .02069, df = 6.0000					
	Var1	**Var2 - Mean**	**1**	**2**	**3**
6	-S3	2.580847			****
5	-S2	3.471365	****		
4	-S0	3.732117	****		
1	+S0	3.732117	****		
3	+S3	3.948937	****	****	
2	+S2	4.456173		****	

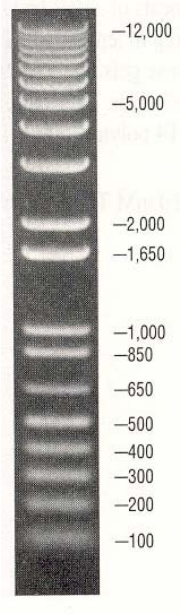
Supplementary data IV – Molecular marker

Molecular marker "1 Kb Plus DNA Ladder"

1 Kb Plus DNA Ladder™ (Invitrogen)	48 µL
10x Blue Juice™ (Invitrogen)	96 µL
TE	336 µL

The products were mixed in a 1.5 mL Eppendorf tube and vortex. The molecular marker was kept at 4 ° C.

Molecular marker "1 Kb Plus DNA Ladder" (Invitrogene) after resolving in 0,9% agarose gel.



Supplementary data