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# Functional Analysis Of Grapevine STS7 And STS22 Promoters

Lianhua Lianhua Xu

# FUNCTIONAL ANALYSIS OF GRAPEVINE *STS7* AND *STS22* PROMOTERS

A Masters Thesis

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Master of Science, Plant Science

By

Lianhua Xu

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**FUNCTIONAL ANALYSIS OF GRAPEVINE *STS7* AND *STS22* PROMOTERS**

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## ABSTRACT

Stilbenic compounds are a group of phytoalexins that are produced by a limited number of plant species including grapevine to defend against diseases. Stilbene synthase (STS) is the key enzyme that catalyzes the biosynthesis of stilbenic compounds. Previous results indicated a significant increase in the abundance of transcripts of *STS7* and *STS22* genes in powdery mildew-infected Cabernet Sauvignon leaves. I isolated the promoter sequences of *STS7* and *STS22* from grapevine *Vitis aestivalis* 'Norton' (Va) and *Vitis vinifera* 'Cabernet Sauvignon' (Vv) and studied their activities in transgenic plants. The results showed high activity of *VaSTS7* and *VaSTS22* promoters in transgenic plant leaves at all developmental stages. *VaSTS22* promoter was activated mainly along the veins, whereas *VaSTS7* promoter was activated in leaf tissues in transgenic plants. Both *VaSTS22* and *VvSTS22* promoters showed higher activity than *VaSTS7* promoter in transgenic plant leaves when treated by salicylic acid. The activity of *VaSTS22* promoter increased in transgenic plant leaves at 10 days post inoculation with powdery mildew, but neither *VaSTS7* nor *VvSTS22* promoter showed significant changes in transgenic plants after inoculation. These assays demonstrated that *STS7* and *STS22* promoter differently regulated a reporter gene in roots, leaves, and also in response to salicylic acid and powdery mildew in transgenic plants. My results provided new knowledge on the involvement of *STS* genes in defense against biotic and abiotic factors.

**KEYWORDS**: plant defense system; powdery mildew; *Botrytis cinerea*; stilbenic compounds; stilbene synthase; promoter

This abstract is approved as to form and content

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Dr. Wenping Qiu
Chairperson, Advisory Committee
Missouri State University

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Dr. Wenping Qiu

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Dr. Anson Elliott

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Dr. Chin-Feng Hwang

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Dr. Julie Masterson, Graduate College Dean

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# CHAPTER1: LITERATURE REVIEW

## Plant Defense System

In plant science field, it is commonly accepted that there are two branches of plant innate immune system to defense against pathogens, one is known as PTI and the other is ETI (Dangl et al. 2013). PTI is an abbreviation of PAMPs-triggered immunity. The PAMPs stand for pathogen-associated molecule patterns. Meanwhile, ETI means effector-triggered immunity. Literally, PTI is triggered by pathogen-associated molecule patterns that are produced by pathogen and are recognized receptors from host plants. In usual, PTI is referred as non-specific resistant immune system since it is the first defense layer in plant surface to restrict the extension of pathogens. ETI is the second defense layer working faster and stronger inside a plant cell to fight against the pathogens which successfully suppress the PTI layer (Boller and Felix 2009).

As first defense layer, PTI protects plants from a wide spectrum of disease by using constitutively defensive structures or chemicals, such as waxes on leaves, rigid cell walls and hormones (Nürnberger et al. 2004). However, in the long process of co-evolution of plants and pathogens, virulent pathogens have developed various strategies to suppress PTI and eventually invade into host plant cells, such as produce specific elicitors onto plants (Dangl et al. 2013). Surely plants manage to produce specific effectors to recognize elicitors from virulent pathogen then a specific resistant immune system, so-called ETI is evolved (Qiu et al. 2015). The interactions between the elicitors from virulence pathogens and the effectors from host plants are extensively assumed to be based on the gene-for-gene hypothesis that was proposed by Harold H.Flor in 1950s, it

stated that for each resistance gene (*R* gene) in the host, there is a corresponding avirulence gene (*Avr* gene) in the pathogen for suppressing host resistance (Jones and Dangl 2006). In other words, the Avr proteins (elicitors) in pathogens are responsible for infecting the host plant, and R proteins (effectors) are used to recognize and encounter the Avr proteins to restrict the growth of pathogens. So far, various sets of *Avr/R* gene pairs have been identified from pathogens and plants, such as *AvrPto, AvrPita, Avr-Ml6* and so on (Nürnberger et al. 2004). Most *R* gene in plants are encoding nucleotide-binding leucine-rich (NLR) repeat proteins that are interacting with corresponding specific proteins from pathogens, especially biotrophic pathogens (Gururani et al. 2012). The interaction between elicitors and effectors is the result of natural selection, but the mechanisms of the interaction is not well understood (Jones and Dangl 2006).

Interestingly, programmed cell death (PCD) was frequently observed to be variedly initiated by effectors in penetrated epidermal cells of different plant species when ETI was activated to defense against powdery mildew (Qiu et al. 2015). It's commonly known that the cells number of a highly organized community is regulated by the rate of cell division and rate of cell death. PCD is a process that if cells are no longer needed, they commit suicide by activating an intracellular apoptosis program. Usually, PCD is also commonly referred as to apoptosis (Kerr et al. 1972).

Furthermore, ETI usually passes a threshold for induction of hypersensitive cell death or hypersensitive response (HR). HR is the most common type of PCD that associates with rapid cell death in the areas around the pathogen-infected sites of plants (Coll et al. 2011). The happening of HR usually indicates the events of ETI in plants in response to pathogens. Those events are triggered by the interaction of some sets of cell

signaling. Cell signaling, also known as signal transduction, is the transmission of molecular signals from exterior of a cell to its interior. Signals received by cells must be transmitted effectively into the cell to ensure an appropriate response. One of the most important functions of cell signal transduction is to control and maintain normal physiological balance within the body.

When plants expose to biotic or biotic stresses, they are able to more quickly and more effectively activate defense responses and many of these plants could develop an enhanced resistance to stop a further pathogen attack in the un-inoculated organs. This enhanced resistance in plants is regarded as systemic acquired resistance (SAR) (Baker et al. 1997). SAR has a broad spectrum of resistance, with no specificity to the initial infection since SAR prevents infection by a wide range of pathogen. The signal molecule salicylic acid (SA) is critically required in SAR. Besides SA, SAR is also associated with an accumulation of proteins that related to pathogen (PR proteins) during plant resistance. In order to understand the mechanism of SAR, researchers used Arabidopsis, a model plant, and discovered that the isochorismate pathway is the major source of SA during SAR.

Pathogen-induced resistance in plants is extraordinarily complicated. Scientists worldwide would keep taking every effort to make further understanding of the mechanisms involved.

**Norton and Cabernet Sauvignon**

Grapevines (*Vitis spp.*) are deciduous, perennial plants. Grape yield that is mainly produced from cultivated varieties makes up a big part of the cash fruit production in the

world (Gao et al. 2014). The demand of grapes and grape product is still in the tendency of increase. Grape berries, wine, grape juice and other relevant grape-derived food products are reported to be associated with human health, especially a moderate consumption of red wine is commonly considered to efficiently reduce the incidence of cancer together with cardiovascular disease probably due to the components of health beneficial chemicals in red wine, such as high content levels of phenolic compounds, flavonoids and linoleic acid (Rathi and Rajput 2014). A variety of those health beneficial chemicals in red wine are common groups of phenolic compounds. In other words, grape fruits including berry skins and berry seeds have a high antioxidant capacity to protect human body from the damage caused by free radicals and help reducing a variety of health problems. The antioxidant potency of grapes as well as the antioxidant activities of grape functional components had been extensively proved through different assays including *in vivo* analysis and *in vitro* investigation. Xu et al. treated grape berries of two *Vitis vinifer*a L. varieties with several chemicals and found an obvious correlation of the antioxidant capacity with an increase in phenolic content (Xu et al. 2014). Besides the increased antioxidant activity, the phenolic compounds were also reported to influence some other important wine features such as flavor, bitterness and color (Garrido and Borges 2013). As planting in outdoor vineyards and exposing to a board range of microorganisms spreading in the air, grapevine species are put at a risk of being infected by numerous known or unknown diseases. The disease-resistant ability varies within different grapevine varieties, some grapevine cultivars are granted with strong disease resistances, whereas other could be highly susceptible species. Therefore, as an agricultural and economic important fruit crop, grapevine has been studied for past

decades to achieve further understanding of molecular systems of defending against both biotic and abiotic stresses. Now, the whole grapevine genome has been sequenced from *V. vinifera* via shotgun sequencing of inbred Pinot Nori cultivar (reference genome sequence 'PH40024'). However, it is a big challenge to overcome diseases caused by a broad spectrum of pathogens since the innate immunity is deficient in popularly cultivated grapevine varieties (Romero-Pérez et al. 2000).

Norton, also called as Cynthiana, is a vigorous wine grapevine cultivar of *V. aestivalis* species from *Vitis* genus in *Vitaceae* plant family (Kadir 2005). It is a durable resistant variety in response to various pathogens including grapevine powdery mildew (*Erysiphe necator*), under an unknown mechanism. Norton is cultivated in popularity in the Midwest of US and its genome is commonly considered to be derived from North American grapevine species *V. aestivalis*. It is famous for being the foundation of the grape wine industry due to its wine characteristics in Missouri State, which planting area is up to 128 ha in Missouri (Jogaiah et al. 2013), and it is also grown in Virginia, Arkansas and some other Southern and Midwestern States in the United State America. Unfortunately, Norton is not an ideal wine grape to produce premium wine because of few less desirable flavors coming from fruit compositions, such as high concentrations of titratable acidity, malate, potassium and high juice pH (Jogaiah et al. 2013). Canopy management practices have been implemented to provide an optimal canopy microclimate and to optimize Norton cultivation for reducing the high levels of undesirable fruit compositions mentioned previously (Jogaiah et al. 2013). Norton has been used to cross with various grape varieties in order to generate newly inbred grape

varieties with a disease-resistant background incorporating with better wine quality and taste.

In contrast to Norton, Cabernet Sauvignon is susceptible to pathogens and is one of the European cultivars that are derived from *V. vinifera* and widely cultivated worldwide due to the good quality and flavor of its red wine. Cabernet Sauvignon was reported to have a higher durability than Norton to tolerate high temperature. The photosynthetic activity was observed without fierce fluctuation and growth statement was relatively stable in Cabernet Sauvignon at 35 °C even though the changes of metabolic contents (increased content levels of secondary sugars such as raffinose, fucose and ribulose and a decrease in primary sugars such as glucose, fructose and sucrose) (Hochberg et al. 2015). Xu et al. applied exogenous 24-epibrassinolide (EBR) at the veraison stage of Cabernet Sauvignon berries and identified the significantly increased content level of phenolic compounds in berries and found enhanced antioxidant capacity together higher health benefits in the wine made from those EBR-treated berries (Xu et al. 2014). In a previous work, the result of ferric reducing antioxidant power (FRAP) assay showed that the antioxidant content and capacity was highest in Cabernet Sauvignon berry skin where the resveratrol prevailed rather than the catechin that predominated in all grape fraction (Lutz et al. 2012). The grape juices exhibited very low content of phenolic acids since no grape berry skin were involved in juice products. Therefore, it is reasonable to correlate the high level of antioxidant activity with this specific phenolic compound, resveratrol. The different levels of sensitivity in response to powdery mildew between these two grapevine cultivars, Norton (*V. aestivalis*) and Cabernet Sauvignon (*V. vinifera*), are presumably to be a result of co-evolution between a

plant and pathogens. *V. aestivalis* was originated from North America and exposed to a range of pathogens including powdery mildew and co-evolved together with such pathogens through a long history resulting in a strong pathogen resistance but *V. vinifera* was not under the exposure to powdery mildew during its evolution time resulting in a high susceptible trait.

Some major differences between Norton and Cabernet Sauvignon through comparing the two grape varieties in some aspects are listed as follows. First , the Norton is resistant to a wide range of pathogens while Cabernet Sauvignon is a high disease-susceptible species in response to pathogens; secondly, Norton berries presented a higher level of resistance against the majority of fungal pathogens than that of Cabernet Sauvignon berries during the development process of berries (Ali et al. 2011). In details, the transcript levels of some genes encoding critical enzymes in the biosynthesis of flavonoid pathway, as well as the amount of anthocyanins, were elevated higher in the berry skin of Norton than Cabernet Sauvignon; thirdly, the salicylic acid (SA) content in Norton was detected to have a constitutively high level but low in Cabernet Sauvignon(Fung et al. 2008); fourthly, the malic acid and phenolic acid levels were higher in the ripening berries in Norton than those in Cabernet Sauvignon; fifthly, the tolerated high temperature without damage of health growth for Norton is lower than that of Cabernet Sauvignon.

**Grapevine Powdery Mildew**

*Erysiphe necator* is the causal factor of powdery mildew (PM) disease on grapevine which is originated in North America and then spread into Europe in the 1850s

resulting in dramatic viticultural production decline (Qiu et al. 2015). Therefore, huge amount of fungicides were constantly used which majorly aimed at grapevines in European vineyards (Qiu et al. 2015).

However, application of large quantitative fungicides can be problematic for several reasons. First, vineyard beneficial microorganisms were severely damaged by artificial chemicals. Second, chemicals were always harmful to people who worked in vineyards. Third, chemicals caused an increase in carbon emissions to environmental atmosphere (Qiu et al. 2015). Therefore, it is important and urgent to develop grapevine cultivars with genetic resistance to cut down the usage of those fungicides and achieve resource-conserving and environment-friendly purpose.

*E. necator* is an obligate biotrophic fungus that relies on viable cells to complete life cycle and survival (Fig. 1). The life cycle of *E. necator* is presented in illustration: First, a conidiospore germinates and produces a germ tube to form an appressorium after it lands on the living tissue epidermis. Second, an infection peg is developed from the appressorium downward surface. Third, the infection peg penetrates the cell wall and stick into superficial cells to form a haustorium. Haustorium is a specialized interface where molecules exchange between fungus and host cells happens. Fourth, fungus produces more hyphae after enough nutrients uptaking from cell through haustorium, then hyphae develops more appressorium and go through the life cycle repeatedly (Qiu et al. 2015).

### *Botrytis cinerea* (Gray Mold) on Grapevine

*Botrytis cinerea* is the causal agent of gray mold disease on more than 220 plant species including grapevine. On grapevines, besides the powdery mildew and downy mildew, gray mold is the most devastating fungal disease causing significant losses to grape production. Different from the powdery mildew which is an obligate biotrophic fungal, *B. cinerea* is a necrotrophic fungus that feeds on host cells first and then kills host cells. The phenomenon of hypersensitive response (HR) was observed during the infection of *B. cinerea*, but the precise mechanism involved in this interaction is still unknown. The integral structure of *B. cinerea* consists of hypha and spores. The complete life cycle of *B. cinerea* is elaborately demonstrated (Fig. 2). First, the spores land on fruit through wind, animals or other media; then, the fruit begins to rot, so that spores set into dead fruit area and generate hypha; third, the hypha grows and expands to cover the whole dead fruit surface. At the same time a numerical number of spores are produced on hypha to form the structure called conidiophore. The following steps after the development of conidiophore are classified into two branches of life cycle depending on the different seasons. On one side, in summer cycle, the conidiophore directly recycles the life processes described previously, that conidia locate on fruit surface then go through the life cycle repeatedly. On the other side, *B. cinerea* survives as a dormant situation over the cold winter by developing a dark brown or black tough structure called sclerotia on the surface of infected fruit. When exogenous conditions are fit for growth, the sclerotia would generate, consequently produce the hypha and spores to form the conidiophore, eventually repeats the life cycle unlimited.

*B. cinerea* attacks young grapevine leaves, inflorescences and mature grape berries and influences important wine characteristics such as flavor, bitterness and color (Kelloniemi et al. 2015). A tremendous amount of fungicides have been applied annually to control fungi including *B. cinerea* on grapevine vineyards. However, the vast usage of those chemicals indeed contains potential adverse influences on human society and environments. In a research showed that the treatment of light-emitting diodes (LED) on detached leaves of 'Campbell Early' and 'Kyoho' grape varieties enhanced the resistance of treated leaves to the infection by *B. cinerea* due to the increased abundance of stilbenic compounds and elevated expression of some other defense-related genes (Ahn et al. 2015).

**Stilbenic Compounds**

Plants naturally produce large families of diverse secondary metabolites to defend themselves from a various array of biotic and abiotic stresses that are facing them constantly. Most of such compounds are synthesized in the phenylpropanoid pathway. Phytoalexins are a group of phenylpropanod chemicals which have the antimicrobial activities and functions that are produced to fight against pathogen or herbivore attacks. The definition of phytoalexins were summarized as 'plant antibiotics synthesized de novo after the plant tissue has been exposed to microbial infection, and not preformed or released from pre-existing plant constituents that function as the basis of the resistance mechanism (Bavaresco et al. 2009).

However, some secondary metabolites are restricted to a narrow range of plant species. In other words, specific plant groups produce some particular secondary

metabolites due to functional divergence. Stilbenic compounds are a small group of phytoalexins referring as polyketides and are only produced by a limited number of 72 unrelated plant species (Vannozzi et al. 2012). A big portion of stilbenic compounds are derived from the basic unit trans-resveratrol (3,5,4'-trihydroxy-trans-stilbene)classified as 1,2-diphenlethylene backbone (Chong et al. 2009). Grapevine, pine, peanut and sorghum have the capacity to produce stilbenic compounds (Parage et al. 2012), which is one group of the phytoalexins produced in those plants that plays a role in their response to stresses (Jeandet et al. 2002). Among those stilbene-producing plant species, only grapevine genome had been sequenced. Stilbenic compounds consist of resveratrol, resveratrol glucosides, resveratroloside, piceid, viniferins, piceatannol/astringinin, astringin, pallidol and so on which are constitutively accumulated in various lignified tissues and organs in plants such as stems, roots and seeds but developmentally regulated in some other non-woody parts such as in plant leaves, flowers and berry skins in responding to stresses (Bavaresco et al. 2009). Those stilbenic compounds were predicted to confer some potential beneficial effects on human health. Among those substances, resveratrol is the best understood stilbenic compounds and has been commonly considered to have health benefit in red wine with a moderate consumption to efficiently reduce the morbidity of disease in humans, such as cancer and cardiovascular diseases (Baur and Sinclair 2006). In a word, health benefits of plant stilbenes make stilbenic compounds considerably interesting to researchers.

Usually, stilbenic compounds are constitutively expressed at a low level in some plants, but have a significant increase in accumulation when plants are under stress conditions. Compared with white grape cultivars, red grapes produce a high

concentration of stilbenic compounds in grape juices (Bavaresco et al. 2009). Pinot noir and Cabernet Sauvignon were considered to have higher accumulation of stilbenes, however, disease-resistant grapevine varieties were thought to express larger amount of stilbenic compounds in berries than susceptible grapevine varieties did (Bavaresco et al. 2009).

**Stilbene Synthase**

Stilbene synthase (STS) is the key enzyme in phenylpropanoid pathway when catalyzing stilbene biosynthesis and modification. Stilbene backbone is catalyzed from three malonyl –CoA and one CoA-ester of a cinnamic acid derivative by stilbene synthase (Chong et al. 2009). A summarized process of stilbene biosynthesis is given in Fig. 3. STS is classified in the type III polyketide synthases (PKS) family. Stilbenic compounds are derived from the substrates called malonyl-CoA and CoA-ester. Chalcone synthases (CHSs) function in the flavonoid biosynthesis pathway, and share the substrates consisting of malonyl –CoA and p-coumaroyl-CoA with STS so that produce the common linear tetraketide intermediate (Parage et al. 2012). The synthesis of malonyl-CoA and CoA-esters of cinnammic acid derivatives commonly occurs in mostly higher plants.

The *STS* gene family consists of 48 *STS* genes annotated on the grapevine genome according to the inbred Pinot Noir cultivar PN40024 genome (Fig. 4) (National Center for Biotechnology Information Genome ID: 401) (Schnee et al. 2008). All those 48 genes are classified into 3 groups: group A, group B and group C (Fig. 5). A previous study speculated that the purifying selection may be dominant driving force in the evolution

process of grapevine *STS* family through the dN/dS analysis (Parage et al. 2012). The dN/dS ratio is a genetic parameter that used to measure the selective pressure performed on encoding gene. The dN is the quotient of non-synonymous substitution to non-synonymous sites, and the dS is the ratio of synonymous substitutions to synonymous sites. Summarily, if the dN/dS=1, then the substitutions were mainly driven by natural selection; if the dN/dS >1, then the substitutions were mainly driven by positive selection; if the dN/dS<1, then the substitutions were whereas driven by purifying selectin. Purifying selection is called negative selection as well as the common known natural selection. Until now, it is not very well understood of the reasons for why grapevine expands so extraordinarily large *STS* gene family. Grapevine domestication was considered to have some possible relationships with that expansion. Besides, Sparvoli et al. 2012 hypothesized that the evolution of the *STS* gene family probably shared the same common original gene with anthocyanin gene family then clustered into separated clades via gene duplications and molecular divergence (Vannozzi et al. 2012). Compared with the unique expansion of *STS* gene family in grapevine, until recently, there were only two *STS* genes in peanut, one in sorghum genome, three in Japanese red pine, one in Japanese knotweed, and at least five in Scots pine (Parage et al. 2012). However, the grapevine does not take this numerical advantage of large *STS* gene family to produce a greater quantity of stilbenic compounds than those fewer *STS* gene-containing plant species.

Parage et al. 2012 corrected and completed the automatic structural annotations and discriminated between complete genes, partial genes, and *pseudogenes*. Parage concluded that 32 *STS* genes are complete, five are partial, and 11 are probably

*pseudogenes*. 48 *STS* genes are located on the chromosomes 10 and 16 respectively. In details, chromosome 10 contains 6 *STS* genes that are classed into group A and the whole size of these six *STS* genes is about 91kb. Besides, chromosome 16 hosts 42 *STS* genes that are cataloged into group B and group C. Those 42 genes are of a fairly long size greater than 472 kb. To some extent, there is a high level of conservation in the grapevine *STS* gene family. This conservation can be illustrated from two main facets. On one primary side, a common gene structure was found on all the 32 complete *STS* genes. This conserved gene structure consists of two coding exons whose lengths are 178 and 998 base pair (bp) respectively, and a noncoding intron with a varied short sequence from 136 to 387 bp. The splicing site of this intro in pre-mRNA follows the eukaryotic GT-AG rule in the pre-mRNA splicing proceeds. In some degree, the high conservation of *STS* genes gives rise to a challenge when design primers to amplify specific genes and makes it difficult to investigate the transcript levels of individual *STS* gene. On the other hand, grapevine STS proteins which are encoding 392 amino acids show a high conservation level ranging from 90.3% to 99.7% of identity. In particular, the 32 complete *STS* genes are all translated into proteins that contain 307 amino acids. In addition, the active sites of grapevine STS proteins were reported to contain a conserved structure (Parage et al., 2012). Parage et al selected VvSTS10 protein to construct molecular analysis and found a high similarity between VvSTS10 protein and CHSs and STSs. Some data showed that the Pro-269 is critical for STS evolution (Parage et al. 2012).

During the process of stilbene biosynthesis, STS quickly and efficiently directs the catalyzing of its precursor substrates which are ubiquitously pre-existing molecules in nearly all plants. The substrates of STS are 4-coumaroyl-CoA and malonyl-CoA. A wide

range of different plant species were selected to generate *STS*-transgenic plants over the past years. For example, Hipskind et al generated transgenic alfalfa plants expressing the grapevine *STS* gene that showed an increased resistance to pathogens (Hipskind and Paiva 2000). In contrast to transgenic alfalfa study, Giorcelli et al transferred grapevine *STS* gene into white poplar and detected a higher concentration of the resveratrol-glycoside piceid in transgenic white poplar plants (Giorcelli et al. 2004). Parage et al infiltrated *VvSTS*-containing *Agrobacterium tumefaciens* into *Nicotiana benthamiana* and detected the accumulation of some stilbene derivatives in the leaf extracts of *Nicotiana benthamiana* further confirming previous research results that found an accumulation of resveratrol-glycoside piceid in transgenic kiwifruits and apples (Giorcelli et al. 2004). This transient expression of *VvSTS* also indicated that coding genes are translated into functional STS enzymes (Parage et al. 2012). In summary, it is promising to introduce grapevine *STS* genes into other plant species to generate transgenic plants to increase resistance against disease or to achieve an increase in beneficial stilbenic components in agriculture crops which are lacking STS-related resistant mechanisms (Bavaresco et al. 2009).

*STS* genes are reported to be induced by a wide spectrum of either abiotic or biotic stress factors, such as wounding, UV-C irradiation, ozone, fosetyl-Al, aluminium chloride, methyl jasmonate, benzothiadiazole, salicylic acid, anoxic treatments, and gray mold (*Botrytis cinerea*), downy mildew (*Plasmopara viticola*), powdery mildew (*Erysiphe necator*), berry rot (*Rhizopus stolonifer*)and *Aspergillus carbonarius* (Bavaresco et al. 2009). In addition, climate and grapevine variety in some level influence the accumulation of STS via indirectly impacting the relationship between

pathogens and grapevines (Bavaresco et al. 2009). For instance, the Pinot noir and Cabernet Sauvignon were used as the grapevine species that produced highest level of resveratrol (Goldberg et al. 1996). Brief information about the biotic stresses, gray mold, downy mildew, powdery mildew, is provided in the following.

Downy mildew infection on grapevine triggered expression of over 20 different *STS* genes in grapevine leaves based on the RT-PCR analysis (Richter et al. 2005). *STS* gene family was highly induced by downy mildew after 48 hours of inoculation, but no induction was observed in early infection except for little transcription level of three *STS* genes (Vannozzi et al. 2012). Gray mold on grapevine is caused by *B. cinerea* which seriously reduced grape yield and quality all over the world. Some American grapevine cultivars together with several interspecific inbred grape cultivars are observed to be resistant to *B. cinerea* due to stilbenic compounds and some other groups of phytoalexins (Bavaresco et al. 2015). Bavaresco et al. revealed that under the conditions without infection of *B. cinerea*, the *Botrytis*-resistant grapevine variety contained higher transcription level of STS than susceptible variety in grape berries. However, a rapid increase in accumulation of STS was observed in Castor, a susceptible grapevine cultivar, in response to inoculation of *B. cinerea* and residue of stilbene as well as some phytoalexins even 16 days after *B. cinerea* infection (Bavaresco et al. 2015). Raymond et al. conducted a comprehensive *Vitis* GeneChip analysis to carefully dissect the differences of gene expression patterns between powdery mildew resistant grapevine cultivar and susceptible cultivar in response to powdery mildew. A significant increase in the abundance of transcripts of STS was detected in powdery mildew susceptible grapevine cultivar after inoculation of powdery mildew (Fung et al. 2008). In addition,

another study showed a strongly increased level of transcription of some selected *STS* genes especially in berry skin but very low level in young leaves, indicating the developmentally differential regulation of *STS* genes in response to powdery mildew (Dai et al. 2012).

UV-C irradiation strongly induced the expression of most grapevine *STS* gene family members (Parage et al. 2012). The level of transcription of *STS* gene family was significantly increased within 24 hours under the treatment of UV-C irradiation based on the analysis of mRNA-seq (Vannozzi et al. 2012). With the UV-irradiation on grape flowers and green berries of a certain grape variety, the accumulation of resveratrol induced by UV treatment was reported to positively correlate with the resistant of that variety to defend against either gray mold or powdery mildew (Shiraishi et al. 2010). The correlation was used to screen resistant grape species in response to *B. cinerea and E. necator*.

It is interesting that the *STS* genes were reported to tissue-specifically and developmentally express in different STS-producing plant species. For example, the cell wall was reported to be one of the plant tissues that possessed the highest level of STS distribution (Wang et al. 2010). STS is developmentally regulated though out the ripening process from veraison to maturity in the skins of healthy grape berries (Gatto et al. 2008). Besides in the berry skin, higher level of STS constitutive accumulation was detected in rachis and roots than in stems. Moreover, the constitutive accumulation of nearly whole grapevine STS members was very low even no accumulation in stems together with other some developing grape tissues and organs, such as flowers, buds and developing grape berries (Vannozzi et al. 2012). The results from Vannozzi et al. work also revealed that

both the constitutive transcription levels of grapevine *STS* gene group B and C are lower than that of group A in young leave tissues. Another interesting finding in Vannozzi et al. study was the expression pattern of grapevine *STS* in the processes of aging or senescence, such as in senescing grapevine leaves and in withering grape berries. In their work, the transcript level of STS during the process of berry withering was measured and observed a strongly increased accumulation of STS in the berry pericarp. In other words, the transcript level of grapevine STS is higher in dry grape berry skin than that in flesh berry skin. On the whole, grapevine STS is strongly induced in grapevine young leave, stems, buds, flowers, developing berries and senescent stage tissues (Vannozzi et al. 2012).

**Pathogen Inducible Promoters**

Plant growth and development concurs with the process of various gene expressions in sequence temporally and spatially. Gene expression generally results from a complex signal transduction system which would be triggered by diverse environmental, chemical and physiological factors. A basic gene regulatory system needs input and output signals, and middle steps that links input and output together. In details, input system is made up of signal receptor, while output consists of RNA and protein, and between them is the signal transduction pathway (Katagiri and Chua 1992).

Gene regulation mainly happens at transcription level which is regulated by the cooperation of various *cis* –acting elements and *trans*-acting factors. Plant gene promoter is an upstream region of DNA consequence that is a critic *cis*-acting element that is pivotal in controlling the initiation and regulation of transcription. The promoter is

usually located at upstream of a gene and directs transcription factors to precisely bind into DNA template, activates RNA polymerase, and dictates the transcription efficiency (Lu et al. 2004). For a full promoter, there are several complementary structures of *cis-elements* arrayed on this segment before the start of a protein-encoding gene. Normally, those *cis-elements* consist of a core promoter region, a proximal region and distal region that both are prior to the upstream of the core promoter region. The core promoter is constituted by a TATA box and binding sites for RNA polymerase II. The TATA box, a binding site for the transcription initiation complex, is the typical characteristic of core promoter region. In generally, those regulatory factors that bind promoters are regarded as *trans*-acting factors. In brief, the promoter regulatory level, such as enhanced or suppressed expression, is the result from the interaction between *cis*-acting elements and *trans*-acting factors.

Generally, promoters are classified into four classes on the basis of how genes are expressed: constitutive promoters that direct gene expression in all tissues and all stages of development; tissue-specific promoters whose down-stream genes are activated only in a particular tissue; inducible promoters that regulate gene expression in response to changing environmental conditions and chemical presence; and synthetic promoters that are designed with special features and are expected to possess the desired advanced technological performances.

In terms of inducible promoters, their inducers could be the presence or absence of abiotic or biotic factors, such as light, temperature, alcohol and microorganisms. According to the different source from which elicitors come, those environmental stimuli can be endogenous signals such as plant hormones, or can be external elicitors including

external physical elicitors and external chemical elicitors (Hernandez-Garcia and Finer 2014). As for external stimulating factors, the activity of numerous plant gene promoters could be triggered by a wide range of pathogens. In most cases the phytoalexin synthesis in plant is activated by pathogen elicitors. This process restricts pathogens to grow and spread after the recognition of invading pathogens. Microbe-associated molecular pattern molecules (MAMPs) are assumed to be the most frequently triggers of plant immunity as well as the disruption of homeostasis caused by infections (Irazoqui et al. 2010). It's commonly known that the activity of pathogenesis-related (PR) proteins is activated when plants are infection with a pathogen. As a common and model inducible expression mechanism, the promoter sequences of PR proteins have been isolated from plants such as Arabidopsis and maize. It's showed that the *PR-1a* promoter has also successfully driven the expression of *Bacillus thuringienesis* delta-endotoxin in transgenic plants.

In modern plant technological implication, the most commonly used promoters are those constitutive promoters either came from plant housekeeping genes such as *rice actin*, or from plant viruses such as *Banana streak virus*. Some tissue-specific promoters are also used in some basic researches, such as *Expansin* gene promoter in ripened fruits. In technological reality, high and continuous overexpression of foreign genes driven by constitutive promoters in transgenic plants has been proven to impact undesirably adverse influence on plant growth and development as well as effects from gene expression driven by tissue-specific promoters in unexpected tissues. Ideal pathogen inducible promoters are those promoters whose activities are only dramatically activated with the presence of incompatible pathogen isolates but keep silence or express in no effect level when pathogens are absent. Until very recently, just very limited number of pathogen-

induced promoters had been isolated, such as rice *OsPR10a* promoter, barley *GER4* promoter. The little known knowledge about the information of those promoters makes it uneasy to apply valuable disease resistant genes to actual crop production through currently biotechnology.

Nowadays, there are a large number of transgenic technologies that have been used to construct disease-resistant plant species, such as the application of RNAi. It's currently undoubted that overexpressing resistance genes in plants is a good method in plant transgenic engineering. A potentially efficient way to overexpress the resistance gene is to active corresponding constructed promoters by the recognition between host plants and pathogens. So far, various crops with disease-resistance, herbicide-tolerance and insect-resistance, have been produced and grown. Especially the disease-resistant plant species have been the hottest agricultural research for long time. For some instance of studies from dicotyledonous plants, Belbahri et al. generated transgenic tobacco plants containing an elicitor gene from pathogen which was desired to be driven by a pathogen induced promoter of hsr203J gene and observed an enhanced disease resistance (Belbahri et al. 2001). The result from Kobayashi et al. study showed that the transgenic potato plants that carry the *StCDPK5VK* gene driven by a pathogen-inducible potato promoter designated as *PVS3* promoter exhibited an increased resistance to the near-obligate pathogen *Phytophthora infestans* (Kobayashi et al. 2012). This result was in accord with a previous work that was conducted by Yamamizo et al. which suggested a high resistance in transgenic potato plants which was transferred with this pathogen-inducible potato *PVS3* promoter in response to early blight pathogen *Alternaria solani* and *p.infestans* (Yamamizo et al. 2006). Besides the researches on dicotyledonous plants,

Moreno et al. introduced the *ZmPR4* promoter from maize by which the β-glucuronidase (GUS) reporter gene was followed to monocotyledonous *Oryza sativa* to gain transformational rice generations (Moreno et al. 2005) . The result of GUS assay indicated a high inducibility of maize *ZmPR4* promoter with the infection of fungal pathogens. Therefore, they built the transgenic rice that contained *ZmPR4* promoter driving *afp* (*ATPase family gene*) gene rather than GUS gene. A range of different degree of resistance in response to *Magnaporthe grisea* was performed within those transgenic lines as expected from hypothesis presumed.

Besides the identification of promoters from plants, more and more work are conducted to analyze promoters in details, such as promoter sequence structure, specific DNA motifs of promoter, potential *cis*-elements in promoter etc., to further understand the functions of promoters and dissect mechanisms involved in plant defense system against pathogen disease as well as how the signal transduction pathways contribute to the expression pattern of major disease-resistant genes. For example, the promoter of rice *OsWRKY13,* a member of *WRKY* gene family that works in rice in response to pathogens, had been chosen to be comprehensively analyzed. Two novel pathogen-responsive *cis*-elements, PRE2 and PRE4 as well as other factors that regulate the expression of *OsWRKY13* gene, such as SWIM zinc finger, together with pathogen inducible protein binding sites, were studied pretty well in the past few years (Cai et al. 2008).

Based on the current situation over the world that most agricultural crop cultivars are indeed in the challenges of significant reduction of yield as well as quality every year due to a wide spectrum of fungal diseases, it is urgent and promising to develop the

efficient and environmentally-safe plant genetic technologies to improve disease-resistant performance in crop.

**Tissue-Specific Promoters**

Promoters are able to affect gene expression products and yields. Various promoters from different plant species have been isolated and applied to plant genetic engineering over the past years, including inducible and tissue-specific promoters. Actually, tissue-specific promoters are considered as inducible promoters somehow since tissue-specific promoters are inducible to endogenous or environmental factors. The transcriptional regulation of tissue-specific expression is controlled by several interacting gene regulations. However, information about the dynamic range still needs further understanding from those promoters (Mijakovic et al. 2005). In this way, some synthetic promoters with the ability to accurately control gene transcriptions spatially have been developed (Venter 2007). The classification of tissue-specific promoters is usually based on the particular tissues where the promoters and transgenes are designed to express, such as root promoters, stem promoters, leaf promoters and seed promoters.

In plant genetic engineering, it is common to use those promoters with high inducibility and driven constitutive gene expression such as *rice actin* (Zhang et al. 1991), *maize ubiquitin* (Christensen and Quail 1996), *Banana streak virus* (Schenk et al., 2001) and *Cauliflower mosaic virus* 35S (CaMV35S) promoters (Guilley et al. 1982) whose activity is affected by some unknown physiological and environmental factors (Sunilkumar et al. 2002). In fact, only very limited number of constitutive promoters are truly constitutive promoters that express in all plant tissues and every developmental

stages even though most are isolated from plant housekeeping gens such as *rice actin* and from plant viruses such as CaMV35S (Hernandez-Garcia and Finer 2014). Either the housekeeping genes originated constitutive promoters or plant viruses originated constitutive promoters have some ticklish problems when applied to basic research in genetic engineering. As for the most commonly used CaMV35S promoter, strictly speaking, has become evolutionarily obstructer to transcriptional machinery of host, since it is relatively highly induced in some specific plant sites rather than equally expresses in all plant tissues because of the influence from of multiple tissues-specific elements and became (Lam et al. 1989). Similarly, some undesirable effects and adverse impacts would potentially be conducted on transgenic host plants if some certain stress-tolerant and disease-resistant genes are highly and continuously overexpressed by the strong plant housekeeping genes-based constitutive promoters that are highly active in all tissues and all developmental stages (Hernandez-Garcia and Finer 2014). In contrast to those high constitutive promoters, tissue-specific promoters have an awesome advantage that would theoretically drive gene expression only in certain sites without influence on the other plant parts. The most widely used tissue-specific promoters are seed-specific promoters that are restricted expressed in the stage of seed development, such as the promoters of *Hordein and Glutenin* genes from the grains of cereal that indicate an improvement of quality in crop (Kawakatsu and Takaiwa 2010). In addition, some fruit-specific promoters have been isolated and applied to transgenic plants in order to strength and stress out the dominant qualitative features in fruit, such as *Expansin* promoters, ACC-oxidase, E8 and PG that all of them are enforced expression in ripened fruits (Hernandez-Garcia and Finer 2014).

From the shortcoming insight, it is extensively known that constitutive expression of foreign proteins driven by constitutive promoters in transgenic plants may stress metabolic burden on plants together with GMO safety questions (Conner et al. 2003) and undesirable pleiotropic effects in transgenic plants, such as the alterations of some plant physiological characteristics (Hsieh et al. 2002). Besides, sequence-dependent homologous silencing (transcriptional silencing) may occur when the promoter is highly active (Rocha et al. 2005). Fortunately, transcriptional silencing problem might be mitigated by promoters with little or no homology and undesirable pleiotropic problem might be circumvented by the use of endogenous regulatory regions of promoters with particular developmental expression patterns (Kasuga et al. 2004). In addition, compared to constitutive promoters, tissue-specific promoters probably restrict the expression of transgenes and native only in target tissues, or regulate gene expression temporally, in despite of an inevitably technical problems in real application that a low expression in unexpected tissue (Azuma et al. 2016). As similar to the influences of gene location and the host genotype information on the expression pattern of transgenic genes, the activities of tissue-specific promoters are subject in some degree to the position effect from the native cis-acting elements such as the native enhancers in which located close to transgene sites (Hernandez-Garcia and Finer 2014). Anyway, it is important to identify and isolate more effective tissue-specific promoters to regulate foreign gene expression in precisely special. In fact, researchers are attracted by promoters that have highly tissue-specific activity when induced by abiotic or/and biotic factors.

**Synthetic Promoters**

A Synthetic promoter is a special fragment of artificially synthesized DNA sequence that is designed with certain elements for intended performance of expression patterns that do not exhibit with natural organisms. Usually, a typically synthetic promoter includes a core promoter region, some repeated or different cis-elements on the upstream of core promoter, and controls the expression of downstream reporter genes or inserted foreign genes (Hernandez-Garcia and Finer 2014). Basically speaking, the application of synthetic promoters is a useful tool to analyze signal pathway network. More details about synthetic promoters are illustrated in a previous publish (Rushton et al. 2002).

**Promoter Analysis in Grapevine**

Grapevine (*Vitis spp*) is an economically significant fruit crop worldwide which is highly susceptible to wide spectrum of pathogens, such as *E. necator* (causal agent of powdery mildew), *B. cinerea* (causal agent of gray mold), and *Plasmopara viticola* (causal agent of downy mildew). Grape growers are searching for effective managements to control the diseases that result in significant loss on either grape quality or grape yield. For example, geneticists and breeders have introduced the genetic background of disease-resistant North American *Vitis* species into inbred grape varieties by crossing the North American *Vitis* species and *V. vinifera*-derived grape varieties. However, those breeding programs are time consuming in generating new grape cultivars. With the great development of modern biotechnology, especially the advanced molecular biology, researchers explore the genetic engineering, in addition to the traditional breeding to

modify the genotype of grapevine and improve the favorable characteristics. Nirala et al. transferred the *rice chitinase* gene driven by a promoter of *maize-ubiquitin* into somatic embryos of grape leaves to generate transgenic grapevines, and observed the highly increased activity of chitinase within those transgenic grapevines and an enhanced resistance to powdery mildew (Nirala et al. 2015). Grapevines are highly susceptible to *Agrobacterium vitis*, the causal agent of grape crown gall disease. The grape crown gall disease is very difficult to be efficiently controlled over grapevines in vineyard. To overcome this issue, Zok et al. successfully generated transgenic grapevines containing *virE1* genes aimed to select crown gall disease-resistant transgenic grapevine lines. Seventeen out of selected 26 transgenic grapevines clearly exhibited resistance against crown gall disease (Zok et al. 2012). Hundreds of transgenic Thompson seedless grapevine plants were generated containing *ech42*, *ech33* and *nag70* genes driven by 34S *Figwort mosaic virus* (FMV) promoter, and planted in field. Over 6 years of observation, the selected transgenic plants were verified to express an increased pathogen-resistance both in parent plants and progenies (Rubio et al. 2014). However, those transgenes that were transferred into host plants were under the control of strong constitutive promoters from viruses, and all of them would potentially cause undesirable effects and adverse impacts on transgenic host plants. Besides, in engineered grapevines, there is no deep knowledge about promoters and lack of native promoters that have been characterized and isolated to drive transcription of native genes since the majority of promoters used in the production of transgenic plants were mainly derived from viruses (Yamamoto et al.; Li et al. 2001). Therefore, the identification and characterization of native promoters in grape varieties is a pressing task in the near future.

*Myb14* is a *R2R3 Myb* transcription factor that binds to the *Box-L5* motif located on *STS* promoter during the processes of transcriptional regulations of *STS* in grapevine (Fang et al. 2014). The interaction between *Myb14* and the *STS* promoter was confirmed by using the yeast one-hybrid assay in their work. In addition, they built construct containing *STS* promoter and GUS reporter gene. They found the *STS* promoter could drive the expression of GUS when activated by *Myb14* in the transgenic Arabidopsis (Fang et al. 2014). To further verify if the *STS* inducibility was associated with the transcriptional abundance of *MYB14*, Duan et al. amplified the *Myb14* promoter sequence from a *V. sylvestris* clone and introduced this promoter into a promoter-reporter system. They conducted a set of experiments to assay the promoter-reporter system with UV, downy mildew, JA and SA. The results of those assays confirmed that the *Myb14* promoter was inducible to UV, downy mildew together with oxidative burst, calcium influx, MAPKs cascade and JA, but not to SA (Duan et al. 2016).

Li et al. 2012 amplified promoter fragments in large scale from different grape species, to control the expression of *VvMybA1* gene in pigment-free somatic embryos and investigated the transcriptional activity of those promoters by the histogram analysis of anthocyanin color. The results revealed that 13 promoters out of those 15 promoters distributing over ubiquitin gene family showed even more highly increased inducibility than inducible the promoters that previously reported, and at least three promoters out of these 13 exhibited a high transcriptional activity that almost was close to the activity of 35S promoter (Li et al. 2012). They further found the activities of those promoters were closely associated with the number of *cis*-acting and root-specific elements. Definitely,

their results contributed significantly to the development of native promoters in grapevine.

In another case, Burger et al. 2006 identified and amplified a fruit- and ripening-specific sugar-responsive *mrip1* promoter, of which the fragment size is 2.2kb upstream the gene, from grapevine to drive the transcription of GFP reporter gene in the nectaries of transgenic tobacco. Their results indicated that the *mrip1* promoter directly regulated the expression of GFP in certain ovaries and nectaries in the stage of flower development without the interactions with other *cis*-acting elements, such as stress-responsive and hormone-responsive elements located on *mrip1*promoter region (Burger et al. 2006). In the past decade, the alcohol dehydrogenase *Adh* multigene family from *V. vinifera* was studied very well at transcription level (Tesnière and Verriès 2001). In fact, *V. vinifera* cell was considered as an adaptive and efficient system to study the functions of *adh* promoter (Torregrosa et al. 2015). Sequence analysis showed that distinct promoter organizations could be related to the different performance of fruit ripening (Tesnière and Verriès 2000).

A much more comprehensive and detailed understanding about the function and mechanism of how native promoters are regulating gene transcription in host plants is significant and urgent necessary for the success in agricultural programs of crop improvement for food production. It's still a big challenge to conduct the scientific research about native promoters in grapevine, even though grapevine genome has been sequenced. There remain a great quantity of efforts in finding and investigating more native promoters, especially the constitutive and native promoters for grapevine engineering.

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# CHAPTER2: FUNCTIONAL STUDY OF *STS* PROMOTERS

## Introduction

As a potential biological and prospective biotechnological tool, promoters are considered to be a significant DNA element in plant genetic engineering for improving agriculturally important traits. They are tremendously attractive to researchers in biology because of their crucial roles in regulating gene expression at transcriptional levels. It is possible to control and enhance the expression of transgenes in transgenic plants via the application of specific synthetic promoters after function-associated elements and relevant components are well characterized.

In plant genetic engineering, rice actin (Zhang et al. 1991), maize ubiquitin (Christensen and Quail 1996), *Banana streak virus* (Schenk et al. 2001) and *Cauliflower mosaic virus* 35S (CaMV35S) promoters, which are with high and strong regulatory activities, are commonly used to drive constitutive gene expression in all tissues at all times. Their activities were demonstrated to be exhibited under all kinds of physiological and environmental factors (Sunilkumar et al. 2002). In grapevine, nevertheless, very few constitutive promoters are available that can be applied to control expressions of desired genes either in basic researches or in applied projects. Li et al. (2012) evaluated the activities of selected promoters of different ubiquitin genes in grapevine and found some highly constitutive promoters using the anthocyanin-based color histogram analysis and GUS assay.

However, constitutive expression might potentially lead to some unexpected concerns. It is known that the constitutive accumulation of foreign proteins driven by

constitutive promoters in transgenic plants may bring metabolic burdens on plants together with safety issues (Conner et al. 2003) and lead to undesirable pleiotropic effects in transgenic plants, such as the alterations of some plant physiological characteristics (Hsieh et al. 2002). Besides, sequence-dependent homologous silencing (transcriptional silencing) may occur when the promoter is highly active (Rocha et al. 2005). Fortunately, transcriptional silencing problem might be mitigated by promoters with little or no homology. The undesirable pleiotropic problem might be solved by using the endogenous regulatory regions of promoters which have specific developmental expression patterns (Kasuga et al. 2004). In addition, compared to constitutive promoters, tissue-specific promoters probably restrict the expression of transgenes to target tissues, or regulate gene expression temporally, in despite of an inevitably technical problems in real application that a low expression in unexpected tissues (Azuma et al. 2016). As similar to the influences of gene location and host genotype on the expression pattern of transgenic genes, the activity of tissue-specific promoters are subject in some degree to the position effect from the native *cis*-acting elements such as the native enhancers which is located close to transgene sites (Hernandez-Garcia and Finer 2014). Seed-specific promoters are the most widely used tissue-specific promoters in current plant engineering fields due to the merits of seed qualitative improvement in transgenic crops, such as the promoters of *Hordein* and *Glutenin* gene from cereal seed with the improved traits in grains (Kawakatsu and Takaiwa 2010). For now, researchers place much more emphasis on seeking fruit-specific promoters that can facilitate improving quality of fruits such as enlargement of size, enrichment of nutrients and concentration of phytoalexins. For example, the promoters of *Expansin* genes, promoters of *ACC-oxidase* genes, promoters

of *E8* and promoters of *PG* have been isolated and applied to drive certain transcription in ripened fruits (Hernandez-Garcia and Finer 2014). In fact, some gene families that are related to secondary metabolism were discovered to have an unusual larger expansion in grapevine cultivar than other plants during the processes of analyzing grapevine genome, such as the terpene synthase family and the *STS* family (Jaillon et al. 2007). Vannozzi et al. analyzed the difference of *STS* transcript level in the drying process of berry withering and observed a strongly increased accumulation of STS in the berry pericarp, further proved the previous work that *STS* is highly expressed in the hypodermal cell wall of berry skin during the stages of berry development (Fornara et al. 2008). As a native promoter with potential tissue specific in grapevine, the selected *STS7* and *STS22* promoters deserve special attention to be delicately dissected in further details for the purpose of constructing transcriptional regulation network with more precise control of native genes and transgenes.

Ideal pathogen inducible promoters are those promoters whose activities are only dramatically increased with the presence of compatible pathogens but remain silent or very low expression when pathogens are absent. Until very recently, only limited number of pathogen-induced promoters had been isolated, such as rice *OsPR10a* promoter (Hwang et al. 2008), barley *GER4* promoter (Himmelbach et al. 2010). The little information of those promoters makes it difficult to apply valuable disease resistant genes to increasing crop production through current biotechnology.

The most commonly cultivated grapevines lack of the comprehensive disease resistant systems to defend against a wide spectrum of pathogens because most of those cultivars are derived from the disease susceptible species *V. vinifera* in Eurasian regions.

While the species *V. aestivalis* has developed an incredible disease resistance in response to pathogen stresses. The difference of pathogen-responsive resistance between these two species is thought to be derived from the selection under disease exposures and co-evolution. The *E. necator*, an obligate biotrophic fungus which is the causal agent of powdery mildew on grapevine, is one of the most prevalent pathogens in vineyards worldwide. The multiple *STS* gene family is especially expanded in *V. vinifera* grapevine but the members of this family have different expression patterns under the stress condition of infection by powdery mildew and the treatment of salicylic acid, depending on the source species of grapevine cultivars. As within the disease resistant Norton grape which is commonly considered to be originated from North America grapevine species *V. aestivalis*, most *STS* genes are continuously expressed in moderate level without obvious difference when absence or presence of powdery mildew, but no constitutive expression of *STS* was observed in the susceptible Cabernet Sauvignon species, a cultivar of susceptible *V. vinifera*, under the pathogen-free conditions. Instead, *STS* was induced to rapidly accumulate in high transcript level in Cabernet Sauvignon after the inoculation of powdery mildew (Fig. 6) (Dai et al. 2012).

Some research results could be used as instances to better illustrate the different expression patterns of *STS* in different varieties. Fung et al. used a comprehensive *Vitis* GeneChip to reliably analyze the expression profiles of eight selected kinase-encoding genes including *MAPKK* gene, *EDS1* gene, *WRKY* gene, and *STS* gene in both *V. aestivalis* and *V. vinifera*. They measured the changes of those genes at transcriptional expression levels post inoculation of powdery mildew and before inoculation as well as the abundance of selected gene-encoding transcription between powdery mildew-

inoculated plants and mock-inoculated plants at six time points, indicating that the abundance of stilbenic synthase gene transcript increased significantly in *V. vinifera* in response to the infection of powdery mildew (Fung et al. 2008). Another work confirmed the transcriptome change of *STS* genes in grapevines in respond to the inoculation of powdery mildew. Dai et al. analyzed the transcriptional expression profiles of eight selected *STS* genes in grapevine leaf tissues in response to the inoculation of powdery mildew, including five *STS* genes in group C (*STS*16/22, *STS*13/17/23), three *STS* genes in group B (*STS*8, a same cluster of *STS*7 in group B in the phylogenetic tree of *STS* gene family), by using the quantitative polymerase chain reaction (qPCR) technology at certain designed time points in both Norton (*V. aestivalis*) and Cabernet Sauvignon (*V. vinifera*). The data presented of their work showed four significant results. First, their results directly indicated the obvious transcription changes of selected *STS* upon powdery mildew infection in Cabernet Sauvignon but not in Norton, particularly at 24 and 48 hours post inoculation; Second, the old grapevine leaves were observed to accumulate a higher abundance of *STS* transcripts than that in younger leaves. Besides, a strongly increased expression level of selected *STS* genes was found in the grape berry skin in both Norton and Cabernet Sauvignon; Third, *STS* genes are inducible under the infection by powdery mildew and individual *STS* gens are differently regulated between the disease-resistant species and disease-susceptible species (Dai et al. 2012). However, the exact mechanisms of different expression patterns of individual *STS* genes are unknown. Recently, Kiselev et al. overexpressed the *VaSTS7* gene in transgenic cell cultures' of grape *Vitis amureasis* Rupr. and analyzed the *STS7* gene expression by using RT-PCR. In their research, a dramatically increased expression level of *STS7* was observed in

transgenic grape cells. They statistically found that, in one hand, the resveratrol that accumulated in transgenic grape cells was about three to six times higher than in control cells; in the other hand, other resveratrol productions were also observed to accumulate in transgenic grape cells highly, about four to nine times higher, than in control cells. In addition, there was a 1.4 times increasing in cellular biomass accumulation along with the enhanced expression of *STS7* gene (Kiselev and Aleynova 2015).

In order to understand mechanisms that result in the difference of defense in response to pathogen including powdery mildew in grapevine varieties, a project on function analysis of *STS* promoters is undertaking. To this end, promoter fragments of three grapevine *STS* genes, *VaSTS22*, *VvSTS22* and *VaSTS7*, were amplified from two genotypes of grapevine, Norton and Cabernet Sauvignon. In the phylogenetic relationship analysis of annotated *STS* genes in grapevine genome, *STS7* is clustered into group B and *STS22* in group C. The promoter of *VvSTS7* could not be isolated and sequenced because of unknown reasons. To further analyze these three gene promoters, their sequences were compared with each other. The results showed several different regions between *VaSTS22* and *VvSTS22* promoter sequences, *VaSTS7* and *VaSTS22* promoter sequences, including insertion and deletion fragments (Fig. 7). In other words, the same *STS* genes from Norton and Cabernet Sauvignon, *VaSTS22* and *VvSTS22*, have different promoter sequences. Similarly, the promoter sequences of *VaSTS7* and *VaSTS22*, two individual *STS* genes from the same grapevine variety, are apparently different (Fig. 8).

It is reasonable to come up with a hypothesis that the defense against pathogen is mainly because of the expression level of *STS* genes rather than the functions of genes. Probably, this difference at transcript levels of *VaSTS* and *VvSTS* genes is due to the

sequences of their promoter regions. It still needs to be further analyzed in more details about various *cis*-acting elements that are located on *STS* promoter regions, such as certain function of individual *cis*-acting element together with corresponding transcriptional factors involving in the processes of *STS* gene expression. In this project, the promoter activities of the selected three genes to activate transgene expression are evaluated using histochemical β-glucuronidase method (GUS assay). Also, this project aims at verifying the inducibility of these three promoters by powdery mildew in transgenic Arabidopsis, since this inducibility happens in grapevines. Besides the powdery mildew, this project is designed to investigate whether or not these three promoters are inducible by grey mold (*B. cinerea*) in Arabidopsis. In addition, assay was conducted to identify the tissue-specificity of those promoters within Arabidopsis.

In order to validate the difference of expression patterns under the stress conditions within the grapevine *STS* gene family, as well as to further understand the mechanisms of how the transcription of *STS* is regulated, a more detailed analysis of individual members of *STS* gene family is urgently required. Even though various studies have been undertaken and contribute importantly to our current understanding of the functions of grapevine *STS* genes, the analysis of grapevine *STS7* and *STS22* in this project would provide more foundational information for regulation and evolution of grapevine *STS* gene family in response to pathogens, and provide more insights into plant disease immune systems. Basically, this promoter evaluation would be useful for identification of strong inducible promoters. Definitely, the promoter analysis can surely make genetically modified crops much safety in which foreign desired genes are introduced under driving by various functional native promoters. Analogously, the

promoter analysis contributes to the economy in agriculture via improving the efficiency in yielding superior crop plants.

A study showed that the more accurate regulatory of promoter activity, the much higher stability of foreign gene expression in transgenic plants (Hammer et al. 2006). Since a previous work showed that the difference at the activity levels of different ubiquitin genes in grapevine is positively due to the number of positive *cis*-elements together with oppositely because of the root-specific elements, rather than either the presence or sizes of 5'-UTR intron (Li et al. 2012). For *VaSTS7 and VaSTS22* genes, their activities are reported to be evoked by powdery mildew, while the decision mechanism is unclear. It is a good idea to study the correlation between these two grapevine *STS* genes with their 5'UTR intron and it is possible to correlate their promoter activities with their *cis*-acting elements and tissue-specific elements. Therefore, the key point is to clarify the factors that influence the gene expression in order to provide a theoretical basis for the interaction between powdery mildew and grapevine. Furthermore, this analysis provides extraordinary insights into evolution across biological kingdoms.

In this project, transgenic Arabidopsis plants containing *VaSTS7*, *VaSTS22* and *VvSTS22* promoters were constructed to analyze the functions of these three promoters in transgenic plants in response to pathogenic stresses, physical stresses and chemical stresses. The results of this project currently showed that, first, all of these three promoters are highly driving the expression of GUS reporter gene in the whole plant tissues under normal growing conditions, including roots and leaves; Second, the inducibility of these three promoters are divergent when infected by powdery mildew. In details, the activity of *STS22* promoter from Norton is increased by powdery mildew

infection which is different from the previous result of the increased expression of *VaSTS22* gene in PM-infected Cabernet Sauvignon; Third, both *STS22* promoters from Norton and Cabernet could be highly induced in the leaves of transgenic plant under the treatment of salicylic acid (SA), whereas the activity of *STS7* promoter is not increased by SA treatment; Fourth, the *VaSTS22* promoter is mainly active along the veins, whereas the *VaSTS7* promoter is mainly active in the leaf tissues. The findings of this project from basic knowledge of these promoters are only all of a very early step. Nevertheless, this research provides an important perspective on potential functions of *STS* promoters and the development and application of *STS* promoters for a variety of further research projects.

## Materials and Methods

**Arabidopsis triple mutant**. The defense mechanism of a plant in response to non-adapted pathogen is referred to as non-host resistance. The *pen2 pad4 sag101*triple mutant was susceptible to microbes including *E. necator* (Lipka et al. 2005) and was previously used to characterize a candidate nitrate transporter/peptide transporter family member that was upregulated in Cabernet Sauvignon by the inoculation with *E. necator* (Pike et al. 2013). The three *pen2 pad4 sag101* genes are the defense genes *PENETRATION 2, PHYTOALEXIN DEFICIENT 4 and SENESCENCEASSOCIATED GENE 101*that synergistically interact with each other to defend against non-adapted pathogens. Therefore, the non-host resistance in the *pen2 pad4 sag101*triple mutant was compromised and highly susceptible to pathogens (Lipka et al. 2005). Lab previous

results showed that the Arabidopsis *pen2 pad4 sag101*triple mutants were susceptible to grapevine powdery mildew *E. necator* (Gao et al. 2014).

**Constructing transgenic Arabidopsis *VaSTS7-, VaSTS22-* and *VvSTS22-* promoter GUS lines.** The sequences of *VaSTS7* promoter (2,223 bp upstream of the start codon), *VaSTS22* promoter (2,048 bp upstream of the start codon) and *VvSTS22* promoter (2,047 bp upstream of the start codon) were amplified by PCR from *V. vinifera* 'Cabernet Sauvignon' (*Vv*) or from *V. aestivalis* 'Norton' (*Va*) (Table 1) and cloned into the binary vector pKGWFS7.0 containing the reporter genes GUS (Fig. 9). The CaMV 35S promoter was also cloned into the binary vector pKGWFS7.0 containing the reporter genes GUS as a positive control. Those four constructs were previously conducted by Ru Dai in the laboratory (Fig. 10). The primer set used to amplify *VaSTS7* promoter sequence was forward primer: 5'-CACCTCGTCTTTTCAAAAGATGATTTTGC-3'; reverse primer: 5'-GAAGGAAAGAGAAGCGTTCTTGGAG-3', and the primer set used to amplify both *VaSTS22* and *VvSTS22* promoter regions was forward primer: 5'-CACCATCCACGAGCCATGTTCTATTAATC-3'; reverse primer: 5'-GTGTGATGACTACTGAAATCGAAGC-3' (Table 2).

Transgenic Arabidopsis lines of triple mutants (*pen2 pad4 sag101*) were generated by the floral dipping method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* strain GV3101. Transformants were screened on half-strength Murashige and Skoog medium containing 50 µg/ml kanamycin. Single-locus homozygous transgenic lines were selected by scoring for the segregation of kanamycin resistance in the T2 and T3 generations. At least three independent transgenic lines were tested and showed similar expression patterns. The genotype of T1 and T2 generations was tested by

PCR using the primer sets used to amplify promoter sequences. The genomic DNA was extracted from leaf tissues of T1 and T2 generations using the QIAGEN DNeasy Plant Mini Kit. The PCR reaction was run in a 20µl volume. Thermal cycling conditions were following: 94 °C for 1min, 35 cycles of 94 °C for 30 sec, 53 °C for 2 min, 72 °C for 1 min, 72 °C for 10 min.

**GUS (Histochemical β-glucuronidase) assays.** Each plant tissue to be stained was infiltrated with GUS substrate buffer (1mM 5-bromo-4-chloro-3-indolyl glucuronide, 100 mM Tris, pH 7.0, 50 mM NaCl, 0.06% Triton X-100, 1mM Potassium ferricyanide) in a vacuum chamber and incubated at 37°C overnight. 70% ethanol was used to stop the assay reaction and completely clear the chlorophyll in green leaf tissues after the GUS substrate was removed. Pictures for the treated plant samples were taken with a Leica EZ4 HD Digital Stereo Microscope with HDMI LEC MONITOR.

**Treatments of biotic or abiotic factors.** First, the salicylic acid treatment. Transgenic Arabidopsis plants were treated with salicylic acid (SA) by spraying 50 µM SA solution in 0.05% Tween solution. 0.05% Tween was sprayed as negative control. Leaves together with roots were collected at 48 h post treatment and stained with GUS substrate buffer overnight.

Second, the inoculation of *Botrytis cinera*. The grapevine *B. cinera* was maintained on potato dextrose agar (PDA) on petri dishes in a dark environment and at 26°C room temperature. Hyphal tip was sub-cultured on PDA plates to produce spores. About 20 days after *B. cinera* propagation, conidia were collected from PDA plates with double-distilled water (dd$H_2O$), and filtered through cheesecloth. A blood cell counting chamber was used to calculate the concentration of spores in small chambers. Then the

spore solution was diluted with $ddH_2O$ to make a series of gradient concentrations of 100 spores/µl and 200 spores/µl to inoculate onto Arabidopsis leaves. A drop of 5 µl spore solution was applied to six equal sites on upper surface of transgenic Arabidopsis leaves under 12h light / 12h dark at 26°C. Same amount of $ddH_2O$ was used as mock inoculation. Observation of the disease spots was conducted every 12 hours post *B. cinerea* spore inoculation.

Third, the inoculation of Powdery mildew. Grapevine powdery mildew was isolated from naturally mildewed living grapevine leaves. In order to purify grapevine powdery mildew, lightly brush a little of the conidia with a fine painting brush onto the upper surface of a dry fresh sterilized healthy grape leaf on petri dish under the dissecting microscope, then keep plates under 12h light / 12h dark at 26°C. A stack of vigorous conidia can be clearly observed under the dissecting microscope about 7-10 days later. Conidial suspensions were prepared before the dust inoculation of powdery mildew conidia on Arabidopsis leaves. A series of gradient concentrations of the conidial suspensions from 104-106 conidia/ml were made by using 0.05% Tween solution for dilution. Conidial suspensions were inoculated on the upper surface of a healthy transgenic Arabidopsis leaf. Mildewed leaves were collected and stained with 0.05% aniline blue then visualized under the dissecting microscope.

## Results

**Homozygous lines of transgenic Arabidopsis *VaSTS7*-, *VaSTS22*- and *VvSTS22*-promoter GUS lines.** Single-locus homozygous transgenic lines of *VaSTS7*, *VaSTS22* and *VvSTS22* promoters were selected by scoring for the segregation of kanamycin resistance in the T2 and T3 generations. The insertion of transgene in transgenic plants was verified by PCR assays (Fig. 11). In other words, homozygous lines of all transgenic Arabidopsis containing the three promoters from Norton and Cabernet Sauvignon were successfully generated.

**Regulations of *STS* promoters by biotic or abiotic factors.** First of all, the developmental regulation of *STS* promoters. Leaves of three different developmental stages, cotyledon, young leaf and mature leaf, were collected for GUS assays. As showed in Fig. 12, leaves of transgenic plants containing *VaSTS22* promoter showed highest GUS activity after staining, whereas leaves of *VvSTS22* transgenic Arabidopsis indicated the least GUS activity after staining. Interestingly, images of GUS staining of leaves of *pVaSTS22* transgenic plants revealed that GUS mainly expressed along the veins, and expressed in leaves at all these three developmental stages. However, GUS mainly expressed along the major veins in *pVvSTS22* transgenic Arabidopsis and the GUS activity was not as high as that in *pVaSTS22* transgenic plants. In contrast, the expression of GUS was mainly in the leaf tissues in *pVaSTS7* transgenic plants. Therefore, the results suggested that *STS7* and *STS22* promoters probably regulate the expression of *STS* genes differently in grapevine leaf tissues.

Second, the salicylic acid regulation of *STS* promoters**.** As showed in Figure 13, high GUS activity was shown in SA-treated leaves of *pVaSTS22* transgenic Arabidopsis

and *pVvSTS22* transgenic Arabidopsis, especially significant in the SA-treated leaves of *pVvSTS22* transgenic plants. Furthermore, GUS was expressed mainly not only along the veins, but also in entire leaf tissues in the leaves of transgenic plants containing either *VaSTS22* or *pVvSTS22* promoter after they were subject to SA treatment. In other words, promoters of *STS22* genes are inducible by SA treatments. However, no significant difference was observed between SA-treated leaf and mock-treated leaf of *pVaSTS7* transgenic Arabidopsis.

Roots were collected at 48 h post SA treatment and stained with GUS substrate buffer overnight. As showed in Figure 14, roots from *pVaSTS7* and *pVaSTS22* transgenic lines showed high GUS activity, but no significant difference was observed after SA treatment. Besides, the expression of GUS in the roots of *pVvSTS22* transgenic plants was very slightly increased by SA treatment. In conclusion, promoter of *STS22* is inducible by SA in leaves of transgenic plants, but not in roots. However, promoter of *VaSTS7* is not inducible by SA neither in leaves nor in roots of transgenic plants.

Third, the regulation of *STS* promoters by powdery mildew. Homozygous transgenic Arabidopsis leaves were inoculated with powdery mildew spores. Treated leaves were sampled at 0 dpi (days post inoculation), 3 dpi, 5 dpi and 10 dpi. Figure 15 showed an increase in GUS activity in leaves of *pVaSTS22* transgenic Arabidopsis in response to powdery mildew inoculation. However, the GUS activity remained unchanged in leaves of transgenic plants containing neither *STS7* promoter nor *STS22* promoter from Cabernet Sauvignon in response to the infection of powdery mildew. Basically, GUS expressed highly in leaves of *pVaSTS22* transgenic plants. Besides, high GUS activity was showed in leaves of *pVaSTS7* transgenic plants but didn't affect by the

infection of powdery mildew. It is tantamount to say that *VaSTS7* promoter from Norton was probably active in native grapevine tissues and was not inducible by powdery mildew. In assumption, both *VaSTS7* and *VaSTS22* promoters from Norton are assumed to have highly activity in native grapevine tissues, whereas only *STS22* promoter from Norton is inducible by the infection of powdery mildew.

**Discussion**

The central dogma of molecular biology states that the hereditary information in DNA is transcripted to RNA and then translated to protein. Promoter is one of the vital elements of a basic structure of a protein-coding gene. All the individual cells, tissues and organs of a plant share the same genome. The difference between each other is determined by the gene regulation, including transcriptional level and translational level. Moreover, it is the promoter that initiates the transcription of a functional gene. In some degrees, the differentiation of individual cells, tissues and organs is significantly affected by promoter sequences and structures.

Results from previous results in our laboratory showed that the abundance of transcripts of *STS7* and *STS22* is significantly increased in powdery mildew infected *Vitis vinifera* 'Cabernet Sauvignon' but not in *Vitis aestivalis* 'Norton', indicating that the expression of *STS* genes is differently regulated in response to powdery mildew. In this project, promoter sequences of *VaSTS22* (2,084 bp) and *VvSTS22* (2,047 bp) from grapevine Norton and Cabernet Sauvignon respectively, and promoter sequence of *STS7* (2,223 bp) from grapevine Norton were isolated, sequenced, and compared. From the comparison between *VaSTS22* promoter and *VvSTS22* promoter, as well as the comparison between *VaSTS7* and *VaSTS22* promoters, differences were found at several locations of their promoters. Therefore, in this thesis project, I investigated the functions of *STS7* and *STS22* promoters by studying their activities in transgenic plants. The results indicated that the expression patterns of *STS* promoters provide new insights on the regulation of *STS* genes in grapevine development and defense responses.

First of all, transgenic Arabidopsis plants containing either *VaSTS7* or *VaSTS22* promoter from Norton showed a high GUS activity in leaves and roots without any treatment, whereas *pVvSTS22* transgenic lines possessed low GUS activity, indicating that the continuous expression of *VaSTS7* and *VaSTS22* genes in Norton is due to a high basal activity, nevertheless the expression of *VvSTS22* gene in Cabernet Sauvignon is because of the inducibility that is conditioned by external and internal factors.

The responsiveness of these two promoters to SA was investigated to analyze the activity of *STS7* and *STS22* promoters in response to abiotic stresses. The results showed that the activity of both *VaSTS22* and *VvSTS22* promoters were increased in transgenic plants after SA treatment. However, the activity of *VaSTS7* promoter showed no changes in transgenic plants upon SA treatment. Therefore, individual *STS* gene functions differently in plant defense against abiotic stresses. Moreover, none of the roots of transgenic Arabidopsis showed any change in activity, suggesting that the inducibility of *STS* promoter to defense against stresses has tissue specificity, mostly in leaf tissues.

All transgenic Arabidopsis lines were inoculated by powdery mildew spores to investigate the activity of *STS7* and *STS22* promoters in response to obligate biotrophic fungi. The results showed that the *VaSTS22* promoter has an increased activity in transgenic plants at 10 days post inoculation by powdery mildew. In contrast to the response in *pVaSTS22* transgenic plants, the activity of *VvSTS22* promoter remained unchanged in transgenic plants with powdery mildew inoculation. This difference in activity of *STS22* promoters in transgenic plants is in consistent with the expression patterns of *STS22* genes in leaves of Norton and Cabernet Sauvignon when infected by powdery mildew. Besides, the activity of *VaSTS7* promoter is also unchanged in

transgenic plants in response to powdery mildew. Therefore, the *STS* promoters differently regulate the defense level against pathogen.

Interestingly, the *VaSTS22* promoter was mainly expressed along veins but *VaSTS7* promoter mainly in leaf tissues. This may explain why *VaSTS7* promoter showed an unchange but high activity in transgenic plants in resistance to powdery mildew since powdery mildew doesn't infect vascular tissues in plants, but mainly epidermis on leaves.

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Table 1. Promoters and reporter GUS genes of two grapevine *STS* genes that were transferred to Arabidopsis.

| ***STS* gene promoter** | **Grapevine Variety** | |
|---|---|---|
| | *V.aestivalis* 'Norton' | *V.vinifera* 'Cabernet Sauvignon' |
| pSTS7 | pVaSTS7 + GUS | |
| pSTS22 | pVaSTS22 + GUS | pVvSTS22 + GUS |

Table 2: Two sets of primers were designed for cloning *STS* promoters based on grapevine reference genome PN40024.

| Promoter | Predicted length | Primer sets |
|---|---|---|
| STS7 | 2,223bp | F: CACCTCGTCTTTTCAAAAGATGATTTTGC |
| | | R: GAAGGAAAGAGAAGCGTTCTTGGAG |
| STS22 | 2,048bp | F: CACCATCCACGAGCCATGTTCTATTAATC |
| | | R: GTGTGATGACTACTGAAATCGAAGC |

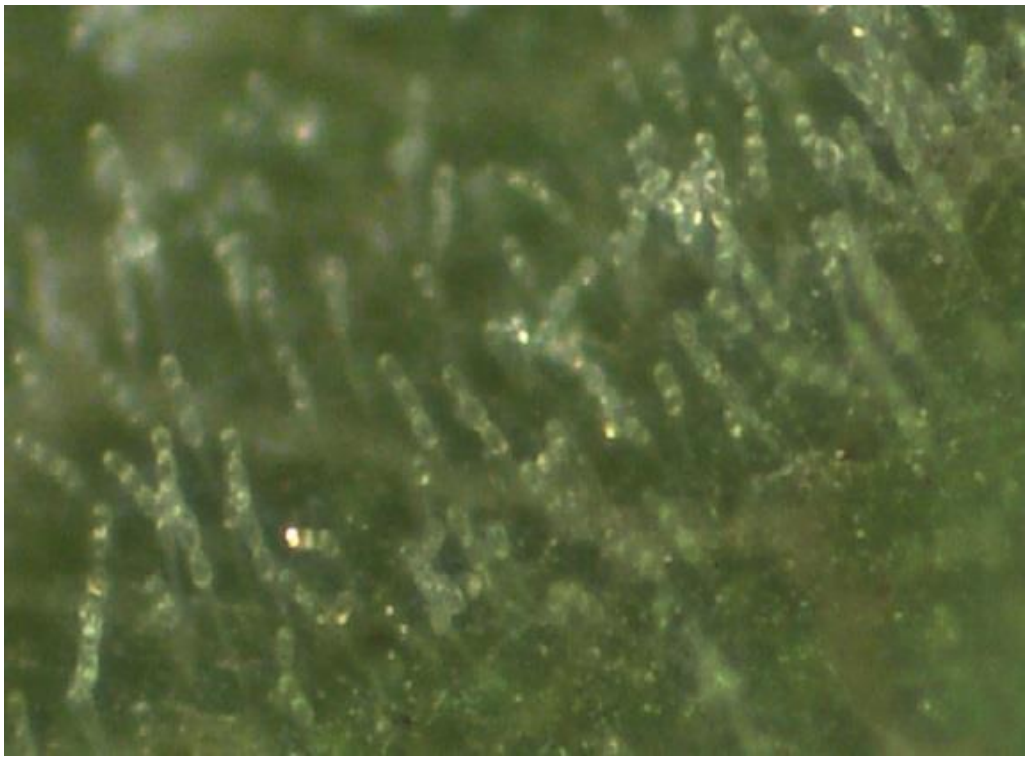

**Fig. 1** Germination processes of grapevine powdery mildew. Conidia of grapevine powdery mildew, formed in chains.

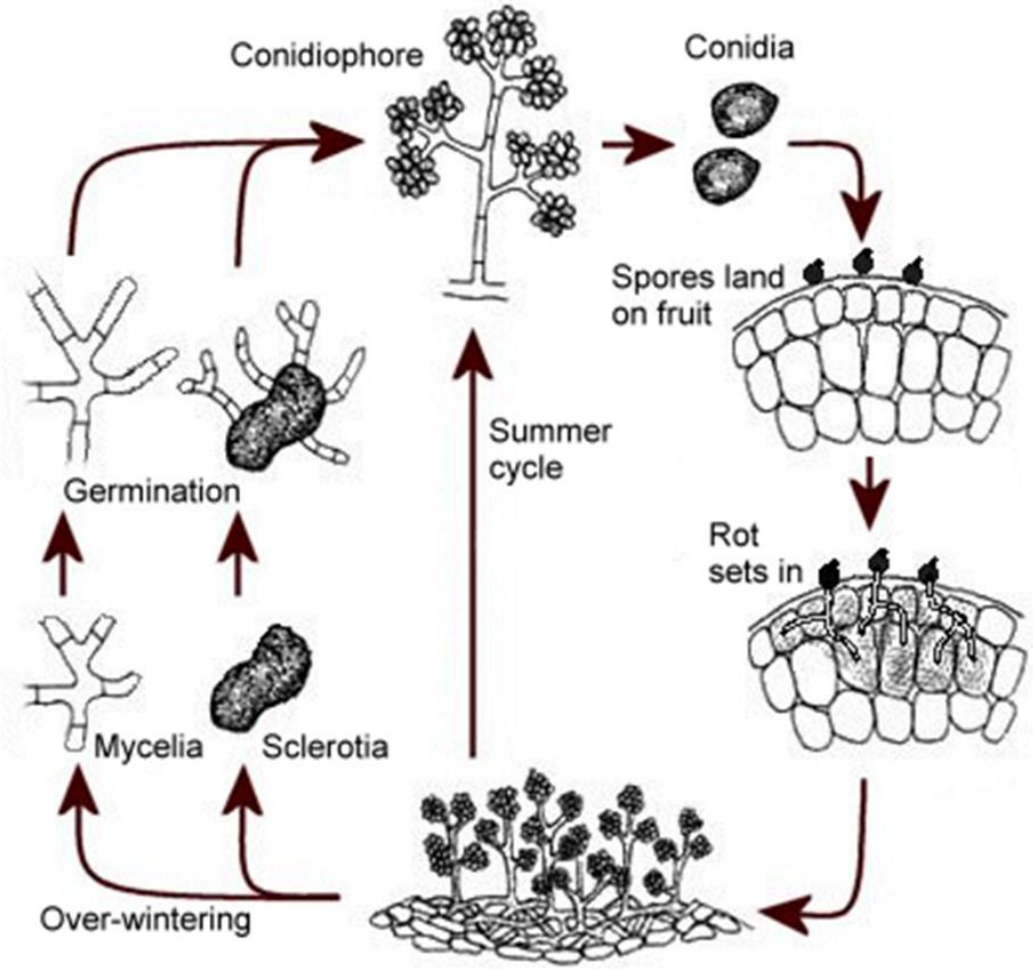


**Fig. 2** Life cycle of *Botrytis cinerea* (https://www.alchimiaweb.com/blogen/botrytis-gray/).

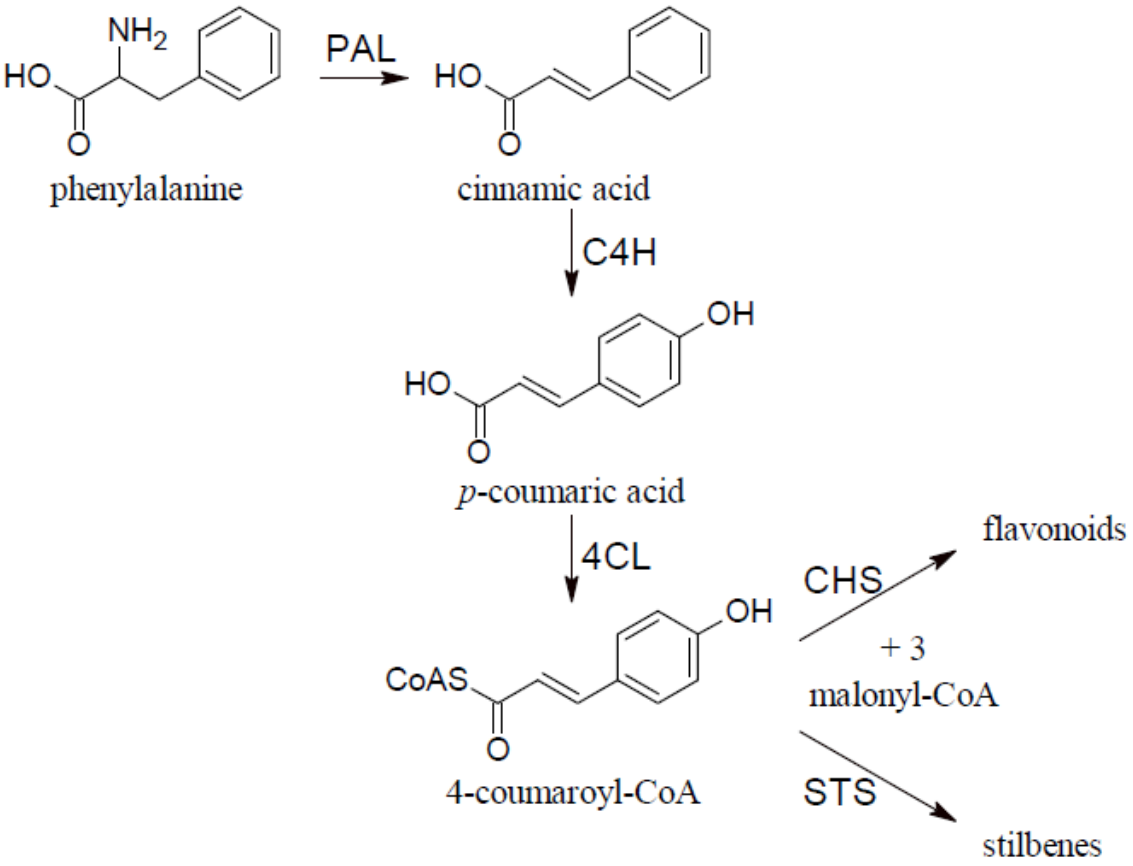


**Fig. 3** Early steps of stilbene biosynthesis. (PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-cumaroyl: CoA-lyase; CHS, chalcone synthase; STS, stilbene synthase) (Chong et al. 2009).


**Fig. 4** *STS* multigene family in grapevine. 48 putative *STS* genes are annotated on grapevine genome PN40024. *STS* genes cluster on chromosomes 10 and 16 respectively. (Parage et al. 2012).

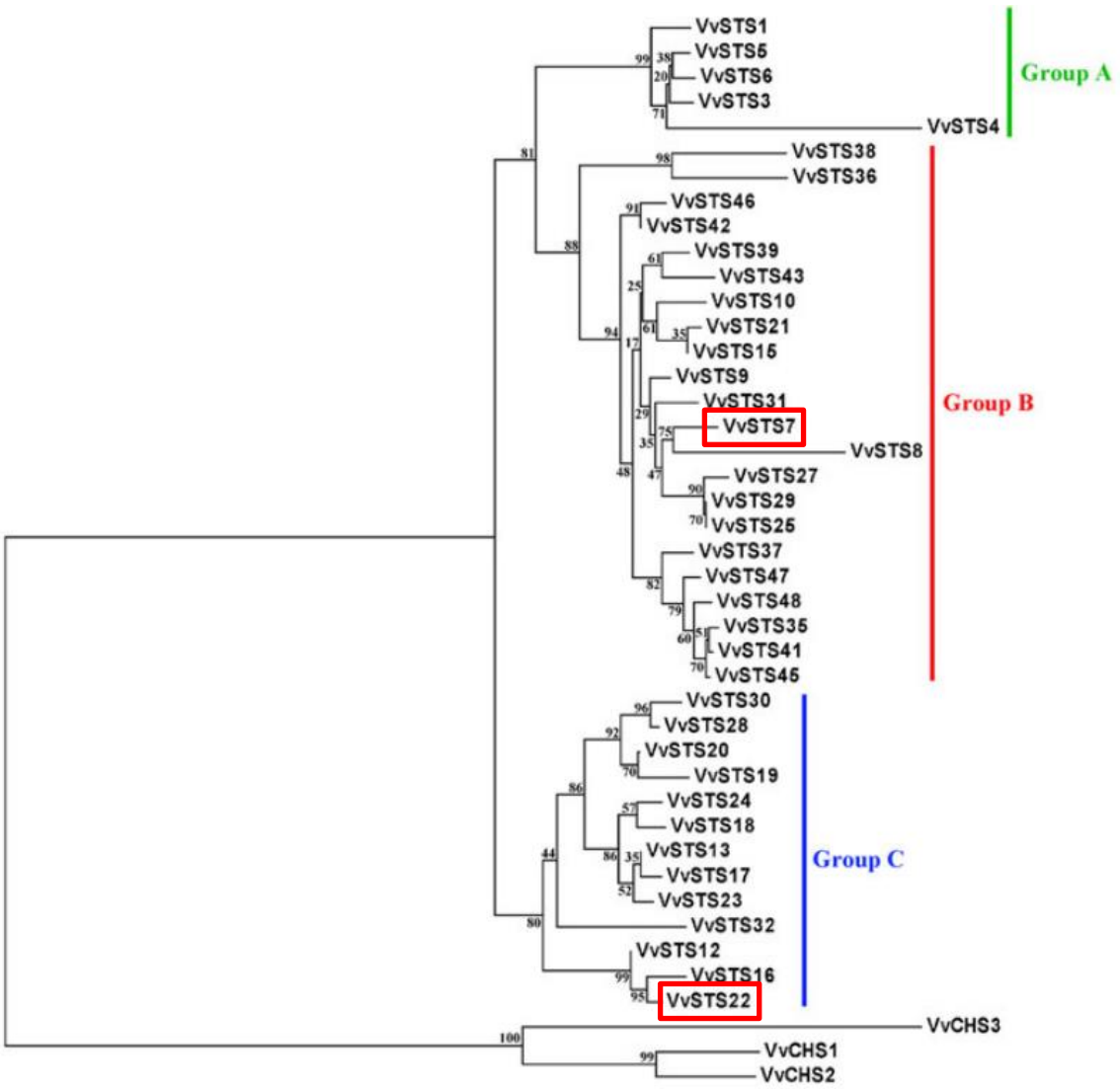


**Fig. 5** Phylogenetic tree of predicted STS proteins in grapevine. Consensus phylogenetic tree generated after sequence alignment with MAFFT 6.0 using the neighbour-joining method. VvSTS gene members predicted to encode for a truncated ORF were not considered. Deduced protein for VvCHS1, VvCHS2 and VvCHS3 were also included in the analysis. Reliability of the predicted tree was tested using bootstrapping with 1000 replicates. Numbers at the forks indicate how often the group to the right appeared among bootstrap replicates. Different coloured bars indicate three main sub-groups designated as A, B and C. (Vannozzi et al. 2012).

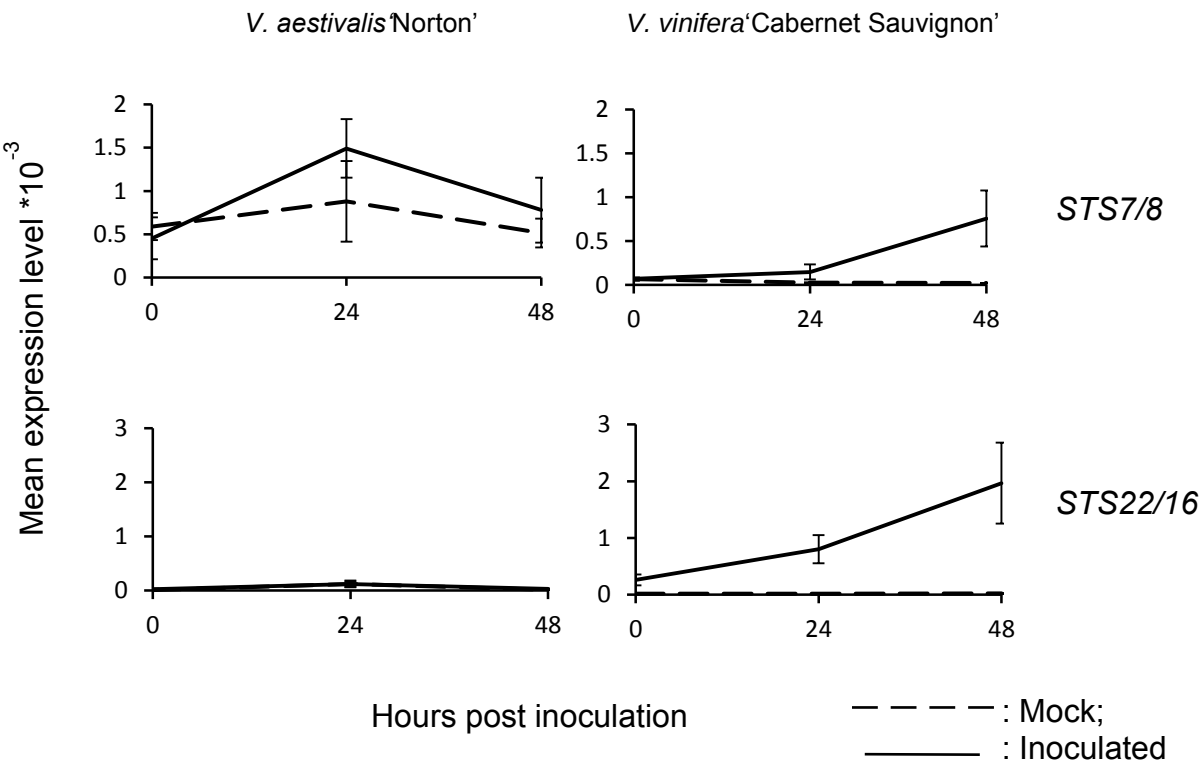


**Fig. 6** Expression level of *STS* genes in PM-inoculated grapevines. Expression level of *STS* genes under the inoculation of Powdery mildew onto Norton leaves (left) and Cabernet Sauvignon leaves (right). Solid line, PM-inoculated samples; Dashed line, mock-inoculated samples. Powdery mildew inoculation highly increased the expression of both *STS7* and *STS22* in the leaves of Cabernet Sauvignon; whereas unregulated expression in the leaves of Norton (Dai et al. 2012).


**Fig. 7** Alignment of *VaSTS22* and *VvSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS22* and *VvSTS22* promoter sequences that are upstreams of transcription initial sites.


**Fig. 7** (Continued) Alignment of *VaSTS22* and *VvSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS22* and *VvSTS22* promoter sequences that are upstreams of transcription initial sites.


**Fig. 7** (Continued) Alignment of *VaSTS22* and *VvSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS22* and *VvSTS22* promoter sequences that are upstreams of transcription initial sites.


**Fig. 7** (Continued) Alignment of *VaSTS22* and *VvSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS22* and *VvSTS22* promoter sequences that are upstreams of transcription initial sites.

```
VaSTS22      GCAGGCAACTTACGTGAAGTGGGTCTCACCTTTCATTTATGGCCTAATGTGCCAACTTTG
VvSTS22      GCAGGCAACTTACGTGAAGTGGGTCTCACCTTTCATTTATGGCCTAATGTGCCAACTTTG
             ************************************************************

VaSTS22      ATCTCCGAAAATATAGAGAAATGTTTGACTCAGGCTTTTGACCCAATTGGAATCAGCGAT
VvSTS22      ATCTCCGAAAATATAGAGAAATGTTTGACTCAGGCTTTTGACCCAATTGGAATCAACGAT
             ************************************************************ ****

VaSTS22      TGGAATTCCTTATTTTGGATTGCTCATCCAGGTGGCCCAGCTATTCTTGATGCAGTTGAA
VvSTS22      TGGAATTCCTTATTTTGGATTGCTCATCCAGGTGGCCCAGCTATTCTTGATGCAGTTGAA
             ************************************************************

VaSTS22      GCAAAATTGAGTTTGGATAAACAAAAACTTAAAGCAACAAGACAAGTTCTAAGTGAATAT
VvSTS22      GCAAAATTGAGTTTGGATAAACAAAAACTTAAAGCAACAAGACATGTTCTAAGTGAATAT
             ******************************************** ***************

VaSTS22      GGGAACATGTCAAGTGCTTGTGTCTTGTTTATTATGGATGAGATGAGAAAGAAATCGTTG
VvSTS22      GGGAACATGTCAAGTGCTTGTGTCTTGTTTATTATGGACGAGATGAGAAAGAAATCGTTG
             ************************************ **********************

VaSTS22      AAGGAAGAAAAAACAACAACAGGTGAAGGATTGGATTGGGGTGTCTTGTTTGGCTTTGGA
VvSTS22      AAGGAAGAAAAAACAACAACAGGTGAAGGATTGGATTGGGGCGTCTTGTTTGGCTTTGGA
             ****************************************** ******************

VaSTS22      CCAGGCTTAACCATCGAGACTGTTGTCCTCCACAGTATTCCTAGGGATTCCAATTGAGTG
VvSTS22      CCAGGCCTAACCATCGAGACTGTTGTCCTCCGCAGTATTCCTAGGGATTCCAATTGAGTG
             ****** ********************** ****************************

VaSTS22      AAAAG
VvSTS22      AAAAG
             *****
```

**Fig. 7** (Continued) Alignment of *VaSTS22* and *VvSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS22* and *VvSTS22* promoter sequences that are upstreams of transcription initial sites.

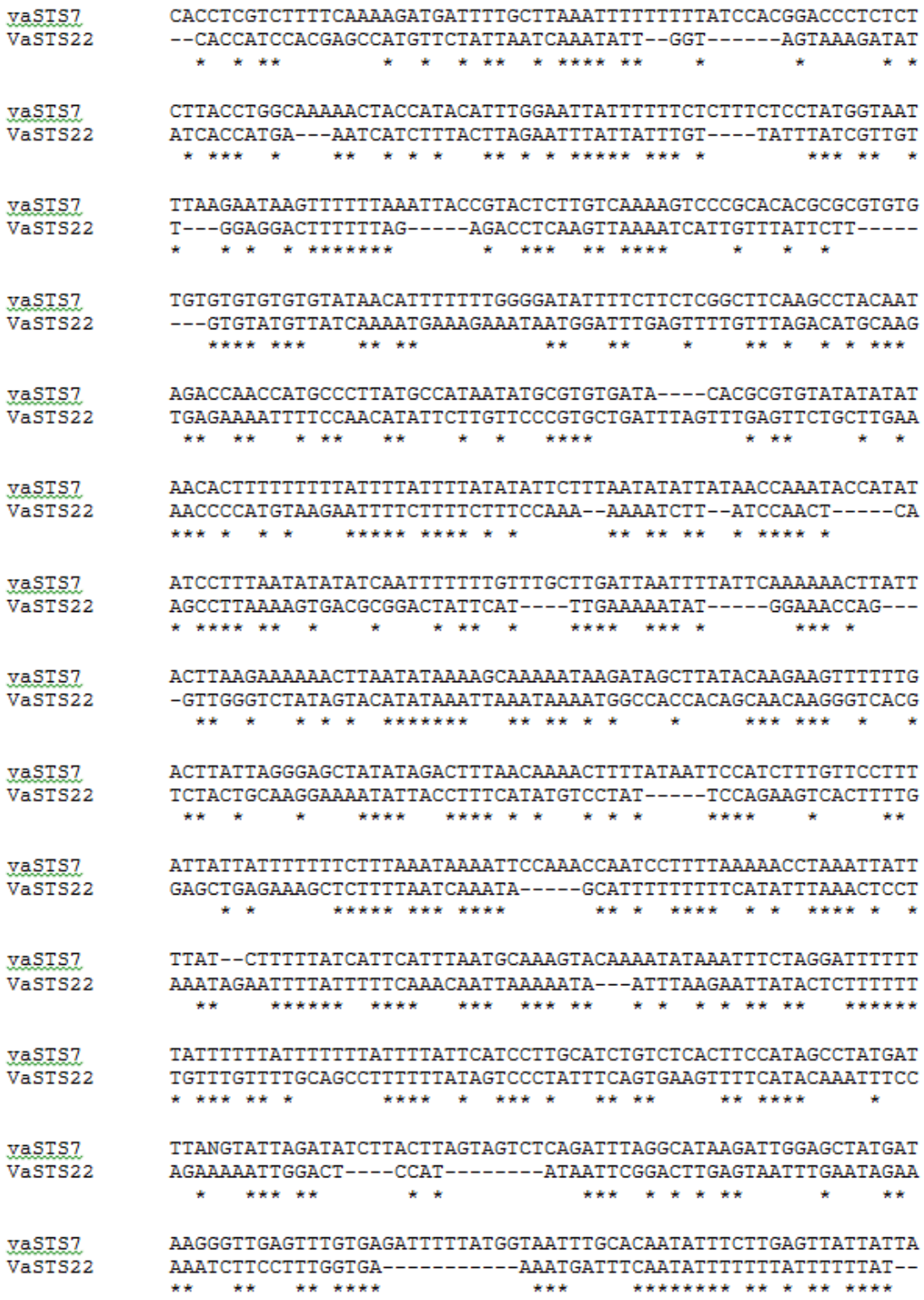


**Fig. 8** Alignment of *VaSTS7* and *VaSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS7* and *VaSTS22* promoter sequences that are upstreams of transcription initial sites.


**Fig. 8** (Continued) Alignment of *VaSTS7* and *VaSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS7* and *VaSTS22* promoter sequences that are upstreams of transcription initial sites.


**Fig. 8** (Continued) Alignment of *VaSTS7* and *VaSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS7* and *VaSTS22* promoter sequences that are upstreams of transcription initial sites.


**Fig. 8** (Continued) Alignment of *VaSTS7* and *VaSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS7* and *VaSTS22* promoter sequences that are upstreams of transcription initial sites.


**Fig. 8** (Continued) Alignment of *VaSTS7* and *VaSTS22* promoter sequences. Fragments in red boxes are represent for the major different regions between *VaSTS7* and *VaSTS22* promoter sequences that are upstreams of transcription initial sites.

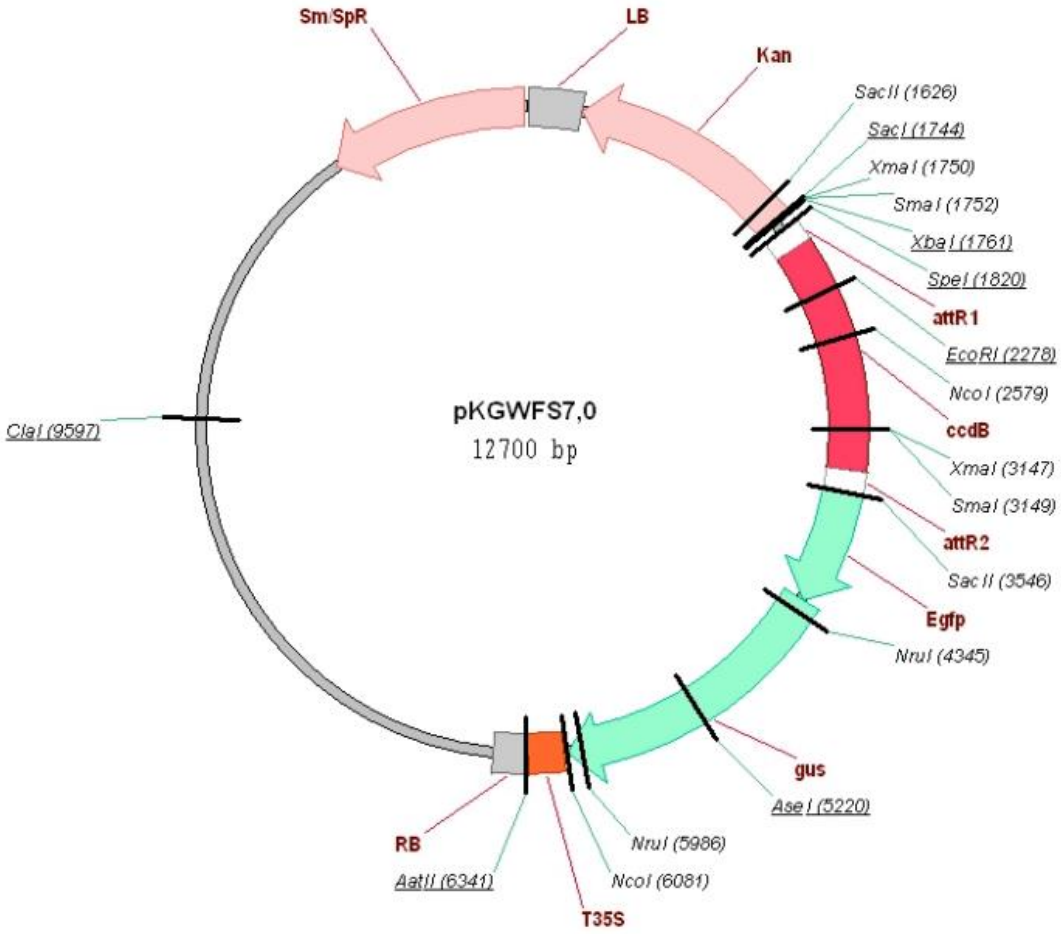


**Fig. 9** Binary vector pKGWFS7.0. Binary vector pKGWFS7.0, containing the reporter gene GUS, Kanamycin and Spectinomycin mark genes, is used to clone *VaSTS7*, *VaSTS22* and *VvSTS22* promoters (http://www.uoguelph.ca/~jcolasan/pdfs/gateway_protocols_and_plasmids.pdf).

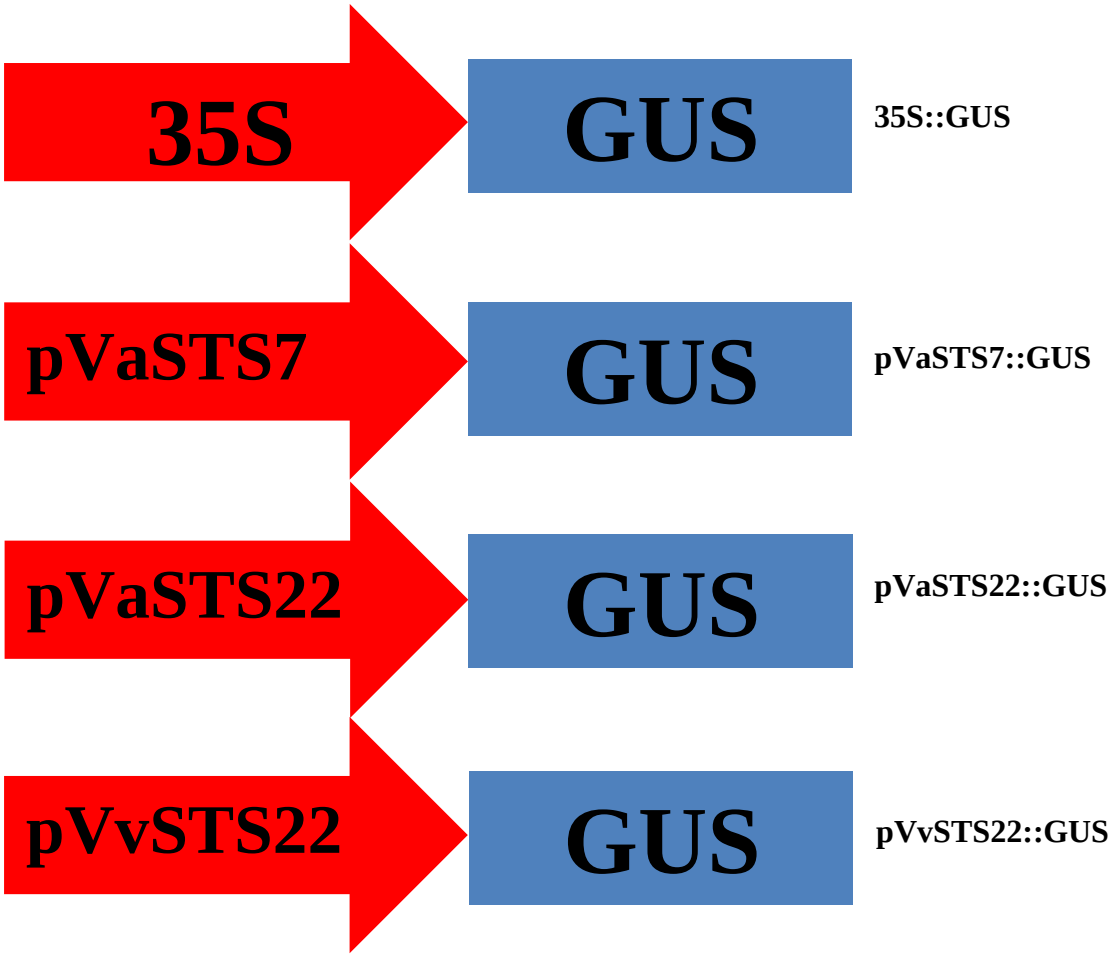


**Fig. 10** Constructs containing promoter and GUS gene. 35S promoter followed by reporter gene GUS is used as positive control. *VaSTS7*, *VaSTS22* and *VvSTS22* promoters are followed by GUS in a construct.

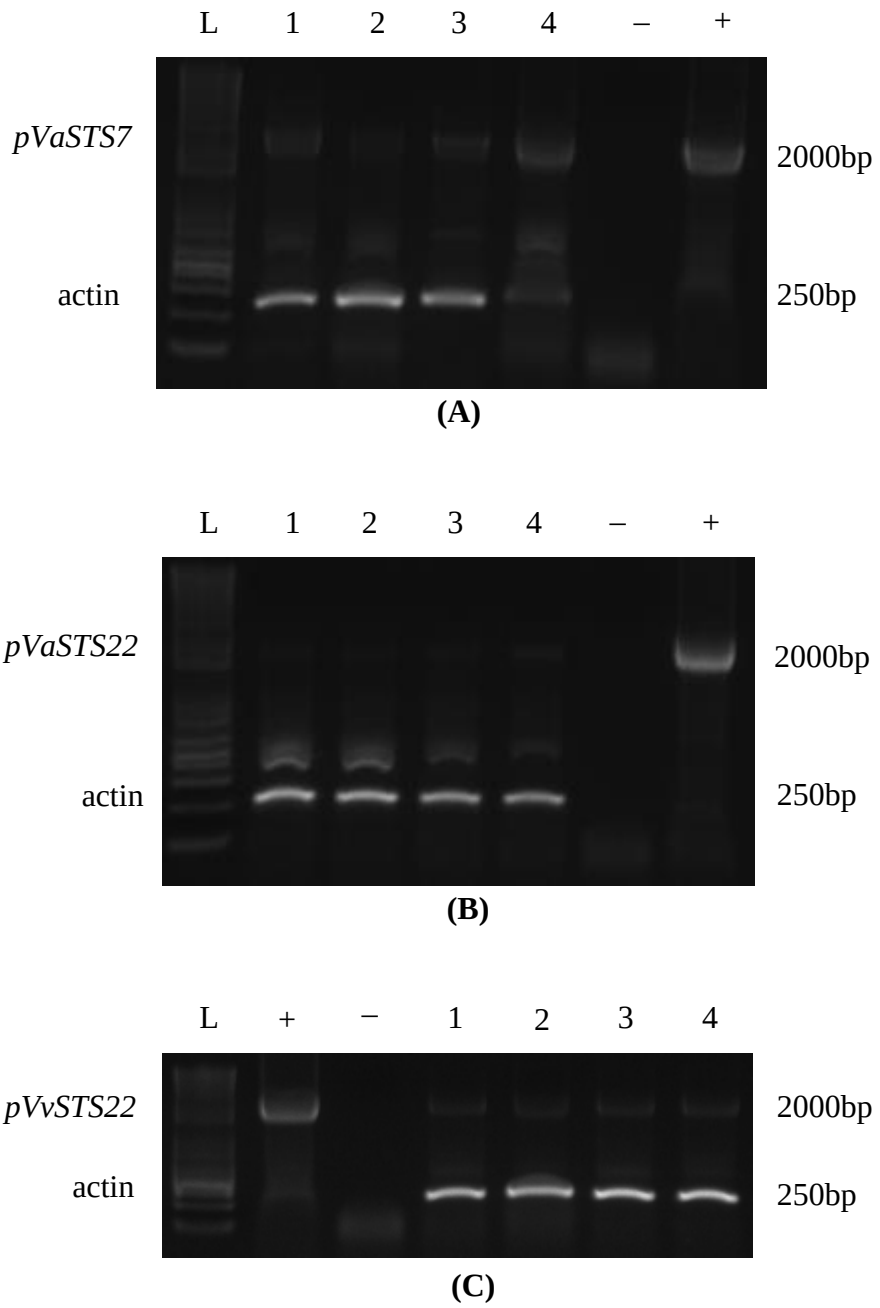


**Fig. 11** Electrophoresis images of DNA fragment amplified using primer sets designed for verifying *STS* promoters in transgenic Arabidopsis. A: *pVvSTS22*; B: *pVaSTS22*; C: *pVaSTS7*. PCR products were analyzed in 1% agrose gel analysis. Ladder is 1kb plus DNA ladder from Invitrogen. Positive and negative control, actin control, four *STS* promoter transgenic lines were included for verification.

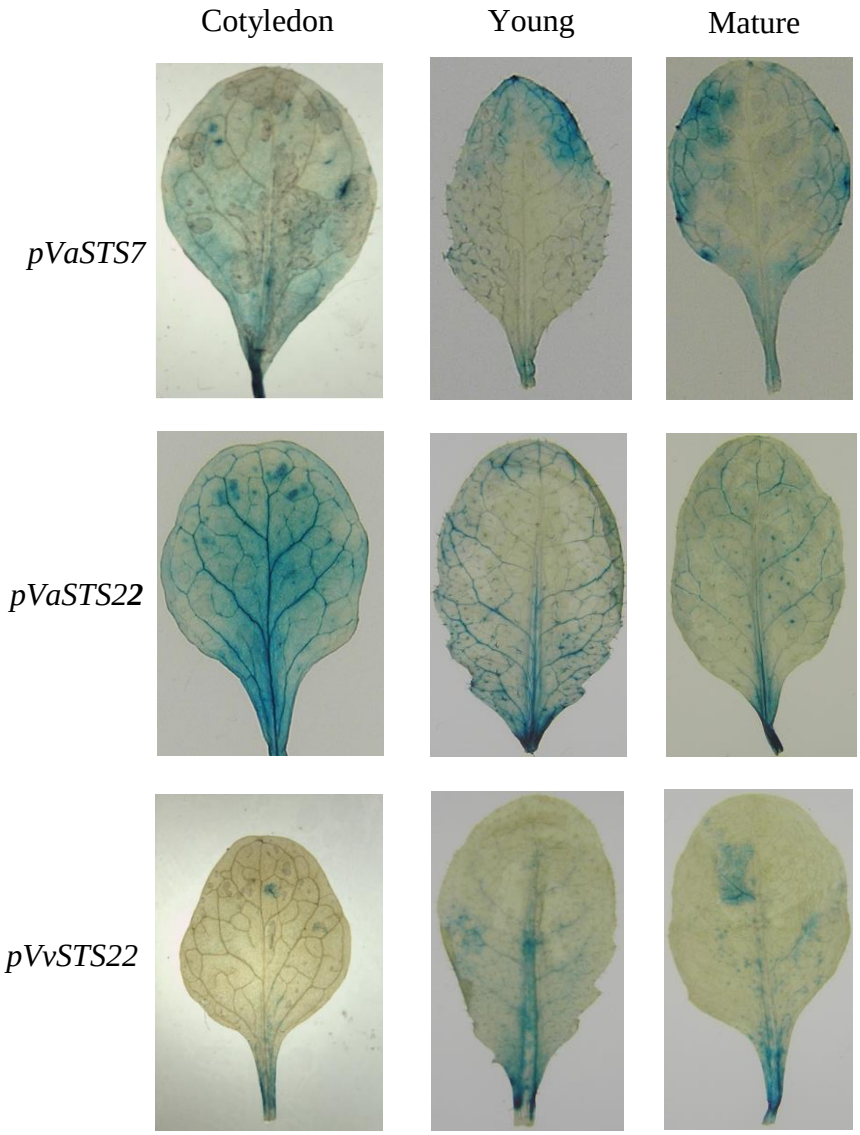


**Fig. 12** Differential activity of *STS7* and *STS22* promoters in transgenic plants. Leaves at three developmental stages, including cotyledon, young and mature leaves, of transgenic Arabidopsis containing either *pVaSTS7*, *pVaSTS22*, or *pVvSTS22* were collected for staining for GUS activity.

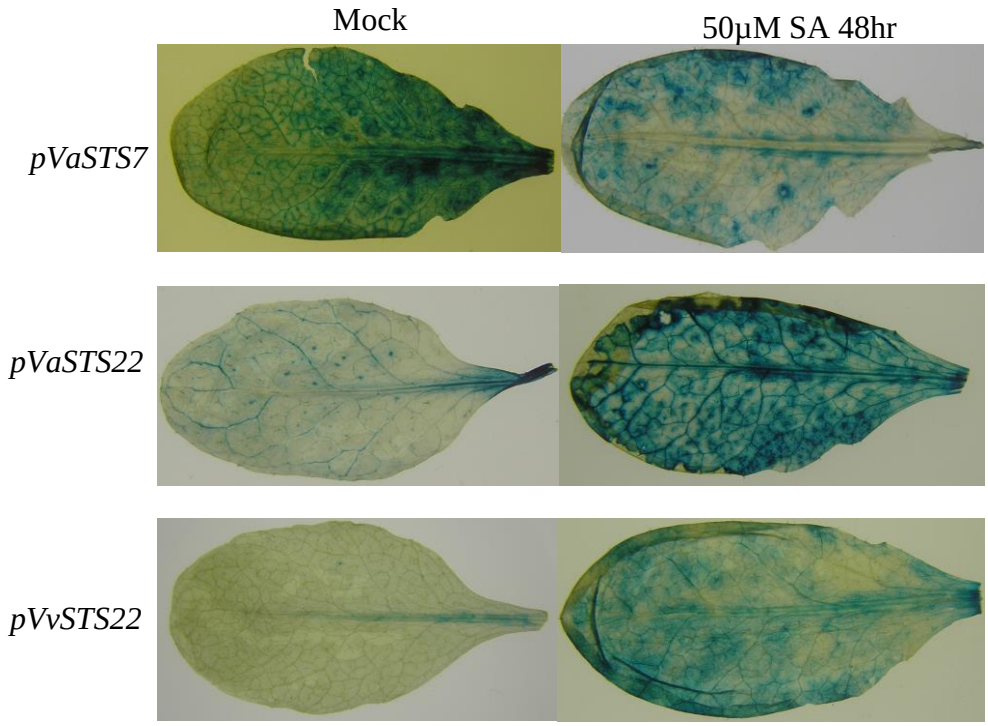


**Fig. 13** GUS assays of leaves of transgenic Arabidopsis under SA treatment. Transgenic Arabidopsis plants containing either the *pVaSTS7*, *pVaSTS22*, or *pVvSTS22*, were sprayed with 50μM SA solution with 0.05% Tween-20. 0.05% Tween-20 was sprayed onto transgenic Arabidopsis leaves as mock treatment. All treated leaves were sampled at 48 h post treatment for staining for GUS activity.

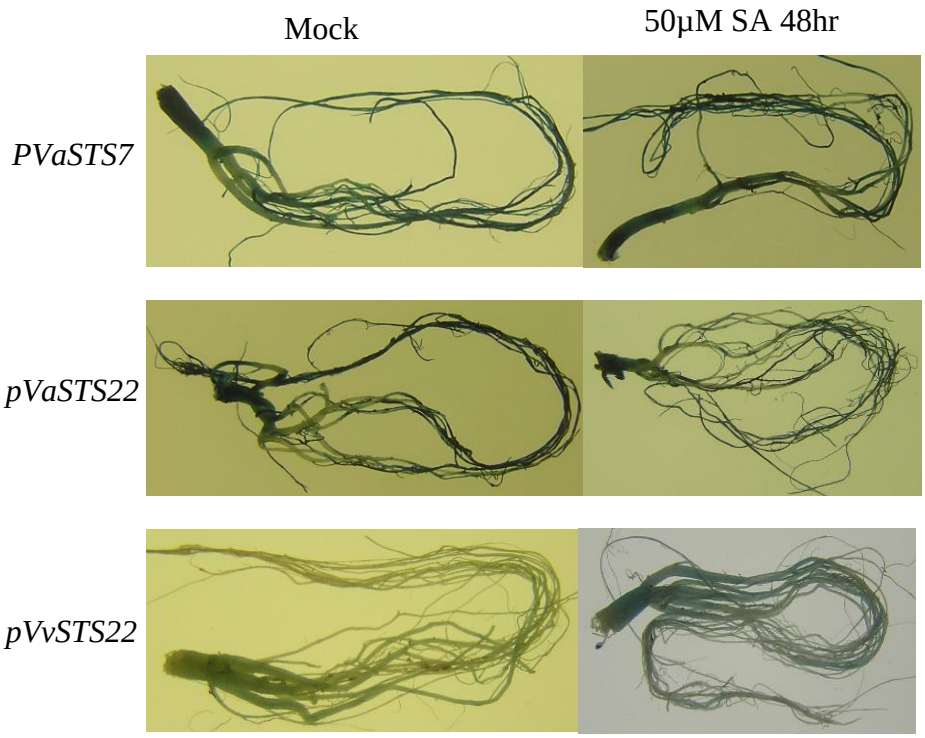


**Fig. 14** GUS assays of roots of transgenic Arabidopsis under SA treatment. Transgenic Arabidopsis plants containing either the *pVaSTS7*, *pVaSTS22*, or *pVvSTS22*, were sprayed with 50µM SA solution with 0.05% Tween-20. 0.05% Tween-20 was sprayed onto transgenic Arabidopsis leaves as mock treatment. All treated roots were sampled at 48 h post treatment for staining for GUS activity.

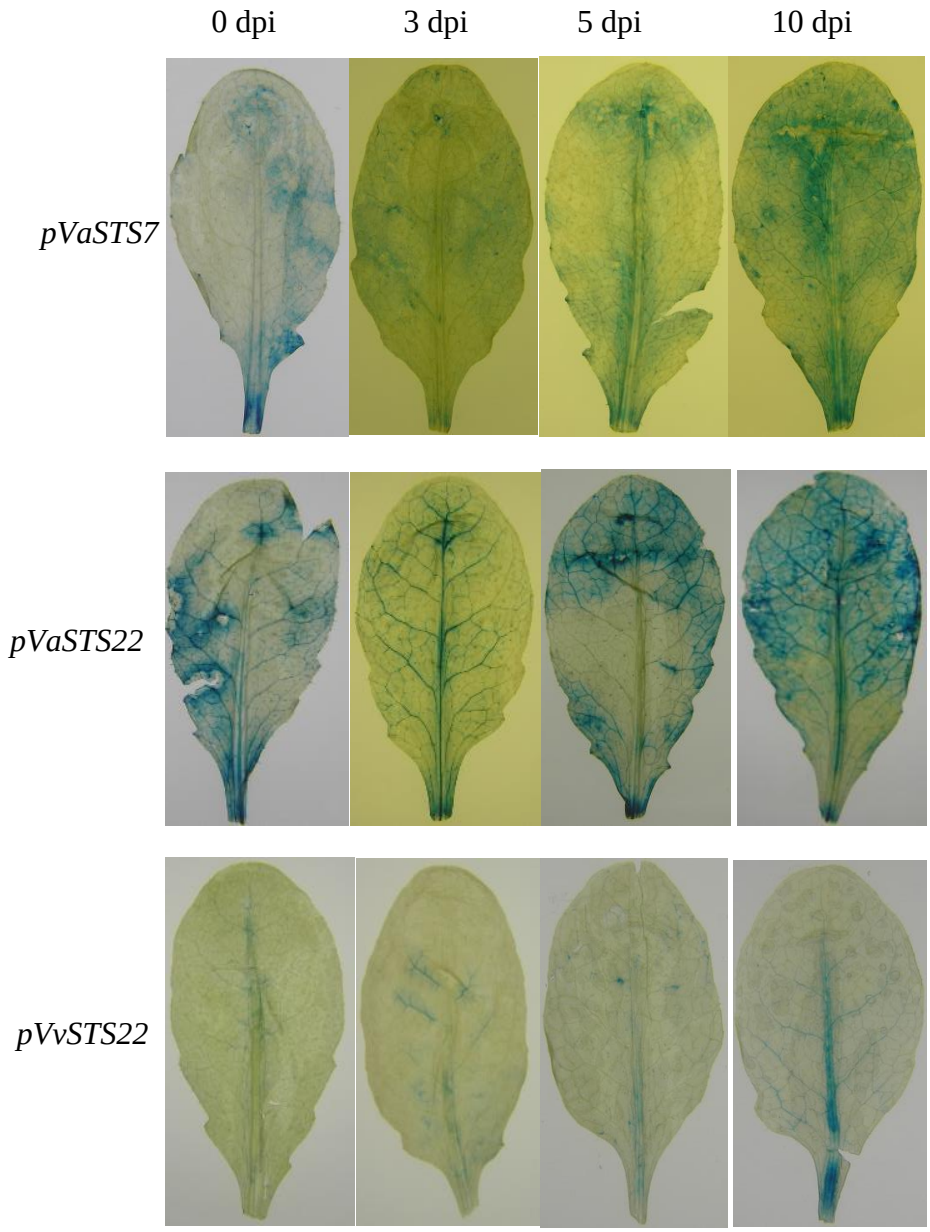


**Fig. 15** GUS assays of leaves of transgenic Arabidopsis under PM inoculation. Transgenic Arabidopsis plants containing either the *pVaSTS7*, *pVaSTS22*, or *pVvSTS22* were inoculated with powdery mildew spores on leaf surface. Arabidopsis leaves were sampled at indicated time points for staining for GUS activity.