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CALCIUM REGULATES *GLADIOLUS* FLOWER SENESCENCE BY INFLUENCING SENESCENCE ASSOCIATED GENES

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SUMMARY

The present investigation was conducted to study the effect of calcium on senescence associated genes and delay in senescence in *Gladiolus* cv. White prosperity. The vase life of flower spikes significantly increased and fresh weight retained for longer duration by $CaCl_2$ (50 mM) treatment over control (distilled water). Calcium treatment maintained higher level of soluble proteins by decreasing protease activity. The expression of senescence associated genes *viz*., *cysteine protease* (*GgCyP1*), *ethylene response sensor 1a* (*GgERS1a*) and *ethylene response sensor 1b* (*GgERS1b*) was down regulated by calcium treatment during the course of flower development. The expression of above genes increased at initial stage and decreased at later stages in both control and treated spikes and the reduction was more prominent in calcium treated spikes over control. The expression of *defender against apoptotic death* (*GgDAD1*) gene was up-regulated by calcium treatment during the course of flower development. On the other hand, the expression of *DAD1* genes increased gradually from I to IV stages and decreased at V stage in both control and treated spikes but the level of expression was higher under Ca treatment. The study suggests that 50 mM calcium treatment may enhance vase life or delay the senescence in gladiolus through increase in soluble proteins, decrease in protease activity, and down regulation of *CyP1*, *ERS1a*, *ERS1b* and up-regulation of *DAD1* genes.

***Key words*: Calcium, *Gladiolus,* gene expression, protease, protein, vase life**

INTRODUCTION

Flowers are highly perishable commodity and after detaching from plants carry on all life processes for their longevity for a few more days at the expense of stored reserved foods. Duration of life of detached flowers depend on the co-ordination of two seemingly conflicting processes, the promotion of growth during the first phase and retardation of senescent processes during the second phase. Senescence involves the ordered disassembly of cellular components in the senescing tissues and allows maximum recovery of nutrients from the senescing tissues for recycling to the parts of the survived plant (Dang *et al.* 2004). Floral senescence in many species *viz*., carnation, *Petunia* etc. is regulated by ethylene (Woltering and van Doorn 1988). However, some species viz., *Gladiolus* (Yamane *et al.* 1993, Serek *et al.* 1994, Arora 2008), *Hemerocallis* (Bieleski and Reid 1992, Valpuesta *et al.* 1995) are insensitive to exogenous ethylene. It remains unclear how the senescence process is initiated and regulated in these species.

Decrease in total proteins during senescence result from increase in proteolytic enzyme activity and decrease in protein synthesis. Up regulated expression of protease genes and enzyme activity with concurrent decline in soluble protein levels occur consistently during senescence in ethylene sensitive (Wagstaff *et al.* 2002,

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Jones *et al.* 2005) as well as insensitive flowers (Arora and Singh 2004, Eason *et al.* 2002, Panavas *et al.* 1999). The expression pattern of cysteine protease varies considerably with respect to the organs of the plant. The SAG 12 from *Arabidopsis* leaves are up regulated from the initiation of senescence (Lohman *et al.* 1994). Treatment of *Sandersonia* and Iris flowers with the cysteine protease inhibitors *viz*., leupeptin and E-64 was found to delay visible symptoms of senescence and decreased endogenous protease activity significantly (Eason *et al.* 2002, Pak and van Doorn 2005, Kant 2008). Induction of Cysteine protease gene expression thus appears to be a common feature of flower senescence (Buchanan-Wollaston 1997, Guerrero *et al.* 1998).

Several studies suggest that senescence of flowers may be triggered by the perception of endogenous ethylene by ethylene receptors. Five ethylene receptor genes have been cloned form *Arabidopsis* and grouped into two sub families (Theologis 1998). The subfamily I comprises ethylene response 1 (ETR1) (Chang *et al.* 1993) and ethylene response sensor 1 (ERS1) (Hua *et al.* 1995). Sub family II comprises ETR 2 (Sakai *et al.* 1998), ethylene insensitive 4 (EIN 4) (Hua *et al.* 1998) and ERS 2 (Hua *et al.* 1998). It was shown that loss of function mutants in each of the *Arabiodopsis* ETR1, ERS1, ETR2, EIN4, and ERS2 did not exhibit defects in ethylene responses. However, triple or quadruple mutants of these genes have constitutive ethylene phenotypes in the absence of ethylene, indicating that these genes collectively regulate ethylene responses negatively (Hua and Meyerowitz 1998). Two ethylene perception paralogous genes, *GgERS1a* and *GgERS1b*, isolated from ethylene insensitive *Gladiolus* flower were found to be homologous to the *Arabidopsis* ethylene receptor gene ERS1 (Arora *et al.* 2006).

The *DAD1* (defender against apoptotic death), a new suppressor gene was identified during studies on a temperature sensitive mutant hamster cell line, tsBN7 (Nakashima *et al.* 1993). *DAD1* is an anti-apoptotic gene. Over-expression of the DAD1 delayed the death of these cells. The function of DAD1 is unclear, it has been suggested that the cell dies due to lack of DAD1 activity because DAD1 is a housekeeping enzyme involved in glycosylation of proteins at ER membranes (Van der Kop *et al.* 2003). Moharikar *et al.* (2007) reported an inverse relationship in the down-regulation of *dad1* with the up-regulation of apoptosis protease activating factor-1 (APAF-1) and the physiological changes that occur in *C. reinhardtii* cells upon exposure to 12 J m^{-2} UV-C. Tanaka *et al.* (1997) reported that *Dad1* is a putative programmed cell death suppressor gene in rice, which showed homology with animal cells *DAD1* and is highly conserved throughout the evolution (Gallois *et al.* 1997, Tanaka *et al* 1997, Silberstein *et al.* 1995, Nakashima *et al.* 1993). Thus, *DAD1* may be a cell survival factor since its cleavage by CED-3/ICE-like protease can trigger apoptosis (Sugimoto *et al.* 1995). In flower petals, thus far only correlative evidence is available between senescence symptoms and *DAD1* expression. Yamada *et al.* (2004) reported that *GlDAD1* mRNA was highly expressed in petals of closed flower buds of *Gladiolus*, but had drastically decreased when the petals had unfolded. The rate of *GlDAD1* expression then remained similar until petal wilting and then became non detectable in fully wilted petals. These results indicate that *GlDAD1* is an anti-senescence gene whose expression is down regulated in the petals before the onset of wilting, the first visible senescence symptom.

Earlier reports from our laboratory have confirmed that sucrose, 5-sulfo salicylic acid, polyamines, trehalose, inositol, α-lipoic acid increase the vase life of *Gladiolus* by delaying the senescence. Calcium (Ca^{2+}) plays a fundamental role in plant membrane stability, cell wall stabilization, and cell integrity (Hirschi 2004). Hung and Kao (2007) reported that calcium chloride and calcium ionophore A23187 effectively reduced H_2O_2-promoted, ABA- or MJ-induced H_2O_2 generation, protein loss, and lipid peroxidation in detached rice leaves. Halevy *et al.* (2001) reported that calcium regulate post-harvest life of flowers. Calcium has also been reported to regulate *Gladiolus* flower senescence by influencing the activity of various antioxidative enzymes and thus minimizing the oxidative stress (Sairam *et al.* 2011). Considering pivotal role of calcium in maintaining plant membrane and cell wall integrity, it is important to investigate the role of calcium in regulating the flower senescence in ethylene insensitive *Gladiolus* flower. The objective of the present investigation was to analyze the expression of senescence associated genes in relation to calcium treatment.

MATERIALS AND METHODS

Plant material and treatments: Experiments were conducted with *Gladiolus grandiflorus* cv. White Prosperity (Family: Iridaceae) raised under recommended cultivation practices. The whole plants were uprooted from the field including rhizome, and brought to lab in large ice buckets. The spikes were cut to a uniform length of 15 cm under distilled water and all the leaves were removed except the bract like leaf below the floret, and placed in test tubes (25 mm dia.) containing 30 ml of vase solution of 1, 10, 25, 50, 100 mmol l^{-1} Ca and control (distilled water). Vase solutions were changed every 24 h and the volume of remaining solution was recorded. The tubes were plugged with non-absorbent cotton to prevent evaporation losses from the surface of vase solution. The tubes were kept at a room temperature of 20±1^{0}C, relative humidity 70±5% under continuous illumination (range 400-700 nm) of 20 Wm^{-2}.

Post-harvest growth of *Gladiolus* in vase solution was divided in to 5 stages, viz., I - tight bud stage, II - half opened stage, III - fully opened stage, IV - incipient senescence stage, V - senescence stage. All parameters were studied at all five stages of flower development. All the determinations were done using flower petals from the third floret from the base of the *Gladiolus* spike of different stages. Petal samples for physiological and biochemical estimations were collected from 4 spikes in duplicate. Data was analyzed for analysis of variance by complete randomized design.

Vase life: Fresh weights of 5 selected spikes were recorded individually daily and per cent increase or decrease in fresh weight compared to that on day zero (time of cutting/harvest) were calculated. Vase life was recorded as the time period for which a spike retained fresh weight similar to that at harvest (Ezhilmathi *et al.* 2007). Based on the results obtained on the vase life, other physiological and biochemical studies were conducted using vase solutions having 50 mmol l^{-1} Ca along with control (distilled water).

Estimation of soluble protein content: For soluble protein content, 0.5 g of petals from 3rd floret at all the stages of flower development were taken and homogenized with 5 ml of 0.1 M phosphate buffer, pH 7.5 using pre-chilled pestle and mortar at 0-4^{0}C. The homogenate was centrifuged for 15 minutes at 10,000 g at 4^{0}C. The supernatant was decanted and equal volume of 15 per cent trichloroacetic acid was added re-centrifuged at 5,000 g for 8 minutes. The precipitate was dissolved in 0.1 N NaOH and volume was made up to 10 ml with 0.1 N NaOH. The protein content was estimated using Bradford's method (Bradford 1976) and expressed as µg protein per g fresh weight of petals.

Assay of protease activity: Total protease activity of the petals was determined following the modified method of Nieri *et al.* (1998) and using azocasein as a synthetic substrate. A crude extract was prepared in 500 µl ice-cold 50 mM TRIS-HCl pH 7.4 and centrifuged at 10,000 g at 4^{o}C. Protease activity of the crude extract was determined by adding 100 µl extract in 200 µl 50 mM Na-acetate (pH 5.0) containing 0.5% (w/v) azocasein. The samples were incubated at 37^{o}C for 24 h and the reactions were then terminated by addition of 50 µl 50% TCA and incubated on ice for one hour. 100 µl aliquots were placed on microtitre plate and alkalinised with 15 µl of 10M NaOH per well and activity was calculated as 1 unit equivalent to a change of 0.01 absorbance units per minute at 492 nm.

Gene expression by RT-PCR: For RT-PCR expression analysis and cloning of cDNAs, the oligonucleotide primers were designed manually, and oligo quality analyzed by using Oligoanalyzer 3.0 tool (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/ ,Integrated DNA Technologies, Coralville, IA 52241, USA).

Total RNA from the petal tissue was extracted by using Trizol reagent (GibcoBRL) as per the recommendations of the manufacturer. DNA contamination was removed from the RNA samples using DNase I (Qiagen Science, Maryland, USA). One µg of total RNA was reverse transcribed using gene specific degenerate primers and Qiagen one step RT-PCR kit. PCR conditions were standardized using gene-specific primers for tubulin. Linear amplification for semi-quantitative RT-PCR was obtained with 35 cycles. Reactions were conducted using My Genie 32 Thermal

Name of the gene	Oligonucleotide sequence	No of PC rcycles	Size of PCR product (kb)	Annealing temp. (^{0}C)
GgCyP1	F-5' ATGCTCCTACTAGCCCTAGTCTTTCTTGC 3'R-5' ACGTAATGCCTCAAATCCACCTCTCTTCC 3'	35	1.40	54
GgERS1a	F-5' ATGGAGGGATGTGATTGCATCGAGCCGCA 3' R-5' CTCCCGACGAGCTAAGTCTAGGGCAACA 3'	35	1.95	58
GgERS1b	F-5' ATGGAGGGATGTGATTGCATCGAGCCGCA 3' R-5' AAATTCATGAACTTGAGGGTCCCTAAAA 3'	35	1.60	55
GgDAD1	F-5' ATGGCAAAATCAACTGCTAAT 3' R-5' TTATCCAAGGAAGTTCATGAT 3'	35	0.31	54
GgAct1	F-5' GGGTGACTCATACTGTTCCAATTTTACGGAA 3' R-5' GCGACCCCCTTAATTTTCATGCTGCTG 3'	35	0.50	55

Block PCR (Bioneer, Korea) under the following conditions: initial PCR activation step: 15 min at 95^{o}C, reverse transcription: 30 min at 50^{o}C, denaturation: 1 min at 94^{o}C, annealing: 1 min at 54 (GgCyP1), 58 (GgERS1a), 55 (GgERS1b), 54 (GgDAD1) and 55^{o}C (GgAct1), extension: 1 min at 72^{o}C, final extension: 10 min at 72^{o}C. The amplification products were electrophoresed on 1.2% agarose gel at 120 volts in TBE buffer (0.4M Tris – borate, 0.001 M EDTA, pH 8.0) using known concentration DNA ladders. RT-PCR reactions were repeated many times to standardize the time and temperature of annealing. Reported gel photograph were selected from the best of three PCR reactions. Gels were stained with ethidium bromide and visualized on Uvi Pro Gel Documentation system (Uvitec, England).

RESULTS

Vase life: Different concentrations of $CaCl_2$ in vase solution extended the vase life of cut *Gladiolus* flowers (Fig. 1). The maximum vase life of 8 days was recorded in 50 mM $CaCl_2$ treatment followed by 10 and 25 mM $CaCl_2$ (7 days), 1 and 100 mM $CaCl_2$ (6 days) and control (5 days). Among all the treatments, vase solutions containing 50 mM $CaCl_2$, was best in extending the vase life of cut *Gladiolus* spikes and was followed by 10 mM, 25 mM vase solution.

Soluble proteins: Soluble proteins in both control and treatment were higher (11.09 mg g^{-1} f.w.) at stage II of flower development (Fig. 2A) followed by control (8.91 mg g^{-1} f.w.) and thereafter, the protein content showed

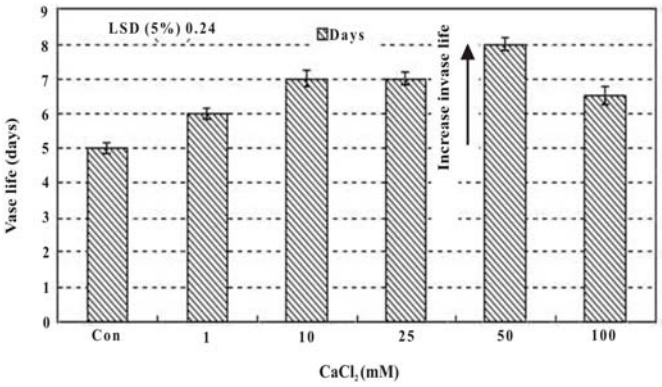

Fig. 1. Effect of calcium treatments on vase life of *Gladiolus* spikes cv. White Prosperity. LSD significant (P ≤ 0.05). Vertical bars show ± SE of mean.

a gradual declining trend till senescence. In general $CaCl_2$ treatment maintained higher soluble protein content over control at all the stages of flower development/ opening. The soluble protein content in $CaCl_2$ treated petals showed an average of 35 % (9.57 mg g^{-1} f.w.) higher than control (7.08 mg g^{-1} f.w.).

Protease activity: Protease activity increased gradually up to III stage of flower opening, and rapidly at IV and V stages (Fig. 2B). Highest protease activity was observed at the V stage of flower opening. $CaCl_2$ treated flower spikes maintained significantly lower protease activity than the flower spikes kept in control vase solution at all the stages. $CaCl_2$ treatment on an average caused 54.3 % decline in protease activity over control.

Gene expression

Cysteine protease gene (GgCyP1): GgCyP1

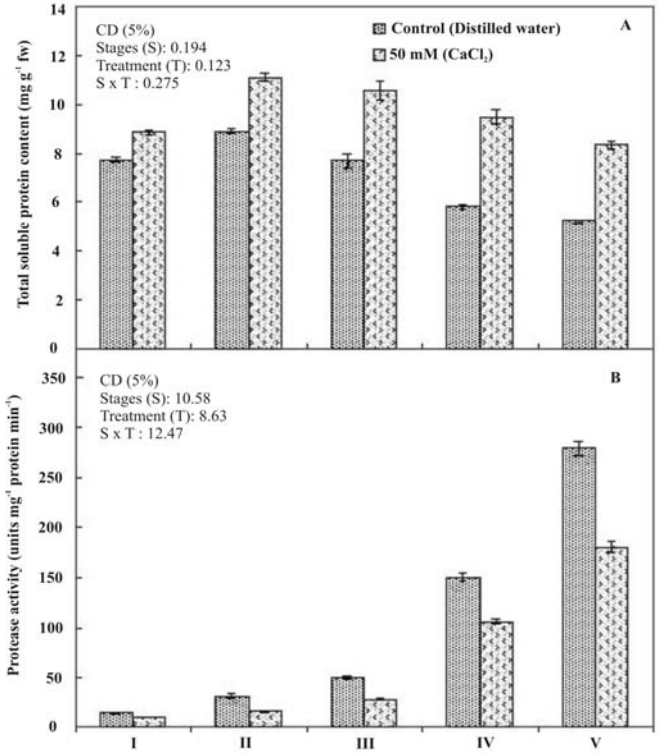

Fig. 2. Effect of calcium treatment on soluble protein content (A) and protease activity (B) in petals of *Gladiolus* cv. White Prosperity at different stages of flower development. LSD significant ($P \leq 0.05$). Vertical bars show ± SE of mean.

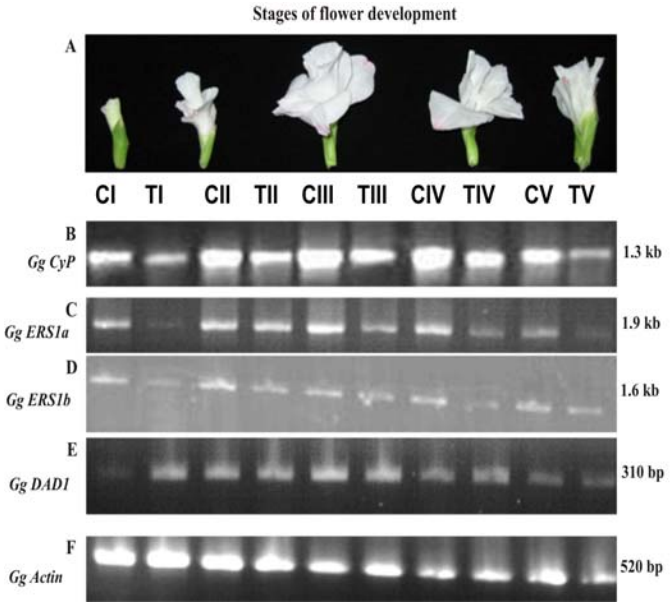

Fig. 3. Various stages of flower opening (A), and expression of senescence associated genes in *Gladiolus*, viz., *GgCyP* (B), *GgERS1a* (C), *GgERS1b* (D), *GgDAD1* (E) and *actin* (F) at different developmental stages in control (C) and $CaCl_2$ (50 mM) treated (T) *Gladiolus* flowers. RT-PCR was performed using total RNA (2 µl) as template with gene specific primers as probe to obtain products representing respective transcripts. *Gladiolus* partial actin gene (*GgActin*) stained with ethidium bromide was used as a control.

expression gradually increased from stage I to IV in both control and treatment (Fig. 3 B). Calcium treatment significantly down regulated the expression of *GgCyP1* at all the stages of flower development. However, there was a significant decline in *GgCyP1* expression at the stage V of treated spikes compared to the control, where the expression was higher than treated spikes.

Ethylene response sensor 1a (GgERS1a): The expression of *GgERS1a* was higher in control than in $CaCl_2$ treated spikes at all the five stages of flower development (Fig. 3C). Expression of *GgERS1a* in control flowers remained almost constant up to IV stage and than declined at V stage, while in case of $CaCl_2$ treated spikes it increased at II stage and than declined and remained almost constant.

Ethylene response sensor 1b (GgERS1b): The *GgERS1b* expression pattern was higher in case of control spikes at all the stages of flower development compared to the $CaCl_2$ treated spikes (Fig. 3D). *GgERS1b* expression in control flowers was almost constant at stages I, III, IV and V, and slightly higher at stage II.

Defender against apoptotic death (GgDAD1): Expression analysis of *GgDAD1* showed that the gene was expressed at all the stages (Fig. 3E). The *GgDAD1* expression increased up to III stage, and $CaCl_2$ treated spikes showed higher expression at all the stages. The expression of *GgDAD1* decreased at stages IV and V, however, $CaCl_2$ treated flowers maintained significantly higher expression at stage IV, while very little expression was observed at stage V in control and treated spikes.

DISCUSSION

The flower petal senescence is mainly associated with the loss of proteins (Woodson and Handa 1987). In this study, there was gradual decline in total soluble

protein and increase in protease activity. Protein degradation is a key feature of disintegration of the membranes, and selective degradation of membrane proteins during senescence is more likely to be achieved through membrane bound proteases, which are present in vacuoles in bulk (Thompson 1974). Lay-Yee *et al.* (1992) reported rapid decline in the protein content in petals of daylily flowers due to very little *de novo* synthesis and considerable net protein degradation. In the present study, it was found that treatments with calcium (50 mM) maintained higher soluble protein content and lower protease activity over control. In rose cut flowers, calcium treatment delayed the decline in membrane proteins and phospholipids in the aging petals (Halevy *et al.* 2001). This suggests that the calcium-induced delay in flower senescence involves protection of membrane proteins and phospholipids from degeneration as well as decline in protease activity.

During senescence, the levels of total RNA decreases, though the expression of genes associated with cell death and senescence increases (Hensel *et al.* 1993, Lohman *et al.* 1994). The identification of genes that expressed specifically during senescence, is the best evidence to show that *de novo* transcription is required for senescence to proceed (Buchanan-Wollaston 1997). In this study of expression of four of the senescence associated genes (SAGs) revealed significant insights into the mechanism of calcium action on gene regulation.

GgCyP1 expression gradually increased from stage I to V in both control and treatment, but the increase was less in calcium treated spikes than control. Thus, the expression of *GgCyP1* was significantly down regulated by calcium at all the stages of flower development. A parallelism was observed between *GgCyP1* expression and protease activity, suggesting that cysteine-protease was the major protease involved in the degradation of proteins during *Gladiolus* flower senescence. A negative relationship exists between *CyP1gene* expression/protease activity and protein content. Delay in senescence by reduced activity of protease has been earlier reported in *Gladiolus* (Arora and Singh 2004), Petunia (Jones *et al.* 2005) and *Alstromeria* (Wagstaff *et al.* 2002). Treatment with the cysteine protease inhibitors *viz*., leupeptin and E-64 delayed visible symptoms of senescence and decreased endogenous protease activity significantly in *Sandersonia*, Iris flowers and *Gladiolus* (Eason *et al.* 2002, Pak and van Doorn 2005, Kant 2008). Cysteine protease gene expression thus appears to be a common feature of flower senescence (Buchanan-Wollaston 1997, Guerrero *et al.* 1998). Since proteolytic activity frequently precedes loss in fresh weight, it might be hypothesized that wilting is driven by increased proteolytic activity during senescence. This is exacerbated by decrease in membrane stability index and lipid peroxidation during senescence, which may result in exposure of proteins to the action of the expressed proteases (Sairam *et al.* 2011).

In the present study calcium treatment increased the vase life and delayed the senescence of cut flowers of ethylene-insensitive *Gladiolus,* and induced reduction in expressions of both *GgERS1a and GgERS1b* compared to control. By implication increase in expressions of these receptors promotes senescence and decline in expression delays senescence. Further $CaCl_2$ treatment, which decreased the expressions of *GgERS1a and GgERS1b,* also decreased the expression of *CyP1* and protease activity, and increased soluble protein content and extended vase life of cut *Gladiolus* flowers. It indicate that these ethylene receptors may positively regulate *Gladiolus* flower senescence by constitutive ethylene phenotypes in the absence of ethylene.

The results on *GgDAD1* expression pattern are consistent with the findings of Yamada *et al.* (2004), where the rate of expression remained similar until petal wilting and then became non-detectable in fully wilted petals. These results indicated that *GgDAD1* expression is down regulated in the petals before the onset of wilting, the first visible senescence symptom. The down regulation of *DAD1* at advance stages of senescence has also been reported in Iris and Carnation (Van der Kop *et al.* 2003). Pea petals treated with ethylene showed a faster decline in *DAD* transcripts levels than untreated controls (Orzaez and Granell 1997). DAD1 has been highly conserved throughout evolution and can be found in plants (Gallois *et al.* 1997, Tanaka *et al.* 1997), yeasts (Silberstein *et al.* 1995) and mammals (Nakashima *et al.* 1993).

Thus the finding of this study concludes that the calcium mediated delay in senescence and increase in vase life of cut *Gladiolus* flowers in terms of physiological parameters might be associated with the down-regulation of the expression of SAGs (*viz*., *GgCyP1*, *GgERS1a* and *GgERS1b)* and up-regulation of the expression of *GgDAD1* during the course of flower development.

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