

DEVELOPMENT OF *AGROBACTERIUM*-BASED TRANSIENT GENE EXPRESSION ASSAY IN ROSE LEAVES

ANEELA YASMIN¹, AKHTAR ALI JALBANI², M. ALI¹, AQEELA NASREEN³
AND THOMAS DEBENER⁴

¹Department of Biotechnology, Faculty of Crop Production, Sindh Agriculture University, Tandojam, Pakistan

²Institute of Information Technology, Sindh Agriculture University, Tandojam, Pakistan

³Centre of Excellence in Marine Biology, University of Karachi, Karachi, Pakistan

⁴Department of Molecular Breeding, Institute of Genetics, University of Hannover, Germany

Abstract

Post-genomics need tools to analyze genomic data for its functional characterization within short time. *Agrobacterium* mediated transient gene expression assays are popular choice to analyze the function of a gene within a few days. These transient gene expression assays have already proved their utility in different plant species. Here we explored the effect of different physical, chemical and biological parameters on transient gene expression in rose leaves using β -glucuronidase (GUS) reporter gene. The selected rose genotypes for this study were *Pariser Charme*, 91/100-5 and 88/124-46 which were maintained in three different pre-culturing conditions i.e. climate chamber, in vitro culture and/ or in tunnels. The leaves of these cultivars were infiltrated with different densities of *Agrobacterium* strain GV3101 and the assay was optimized. The efficiency of this assay was found to mainly depend on rose genotypes, age of leaves, plant pre-culture method and density of *Agrobacterium*. The highest GUS expression was recorded in type B leaves (light green leaves with dark green prominent veins and red edges) of *Pariser Charme* and type C leaves (complete light green young leaves) of 91/100-5 grown in tunnels and when kept at 22°C in dark after agro-infiltration. The optimized bacterial density for the best GUS expression was found to be OD₆₀₀ - 1.5 in simple water without acetosyringone.

Introduction

Rose is the most popular and versatile ornamental. The problems related to its polyploid nature, germination, reproduction and fertility poses serious limitations to characterize this shrub on molecular level. However, during the last two decades valuable genomic resources are generated for this woody plant (reviewed by Byrne, 2009; Table 1). Now-a-days post genomic tools are of great importance to analyze the functionality of the data produced through genomics. Stable transformants are an important source to prove the functionality of a gene. Generating stable transformants is a lengthy process moreover the expression of transgene is effected by many factors as its position in the chromatin and/or gene silencing due to which many transgenic plants are required for the final analysis of transgene. Although it takes 9-12 months to generate stable transformants of roses (Dohm *et al.*, 2002a & b; Marchant *et al.*, 1998 a & b) the rose transformation efficiency is quite low. Alternative to stable transformations is transient gene expression assays which can be performed by either particle bombardment or *Agrobacterium*. In case of *Agrobacterium* mediated stable transformations, it infects the host and integrates its T-DNA to the chromatin of that host. Whereas, transient gene expression is 1000 times higher compared to stable transformants because of the expression of even non-chromatin-integrated T-DNA complexes present in the host cell (Janssen & Gardner, 1989).

According to the findings of Wroblewski *et al.*, (2005) the efficiency of this powerful assay mainly depends on the compatible interaction of bacterium and the host genotype. Different plant species can be rated as sensitive or resistant to agro-infections. This investigation was aimed to develop a gene expression system in rose leaves. In this regard, *Agrobacterium* carrying β -glucuronidase gene were utilized to study the effect of some physical (temperature, light, infiltration method, rose pre-culture method), chemical (acetosyringone, inoculation buffer) and biological factors

(rose genotype, bacterial concentration, age of leaves) on the gene expression.

Material and Methods

Plant genotypes: Three rose genotypes namely, *Pariser Charme* (4x- cultivar), 91/100-5 (4x-experimental hybrid) and 88/124-46 (2x-hybrid) were screened during this investigation for the effect of their genetic background on their interaction with *Agrobacterium*. All rose genotypes belong to the Department of Molecular Breeding, Leibniz University of Hannover, Germany. The rose plants were grown in three different pre-culture conditions. Climate Chamber: 14 hours photoperiod and temperature 20±5 C; *In vitro* culture: Somatic embryos were initiated from callus culture of leaf segments on MS solid basal media and further plantlet regeneration and clonal multiplication was carried out (Dohm *et al.*, 2002a; Chaudhry *et al.*, 2010; Ahmed *et al.*, 2011; Naeem *et al.*, 2013) at 25±1 C and 16 hours light time; Tunnels: cuttings of the selected genotypes were grown in tunnels at 35±5 C with 90-95% humidity. The leaves of different ages from plants were collected and transported to laboratory in humid conditions.

Preparation of *Agrobacterium*: In this study *Agrobacterium* GV3101::pMP90 carrying GUS-intron gene under 35S promoter was used. The expression vector was pBINPLUS (Van Engelen *et al.*, 1995). Bacteria were cultivated in YEP liquid medium for 16-hours at 28 C at 180 rpm. YEP Medium was supplemented with antibiotics Kanamycin (50mg/L), Rifampicin (10mg/L) and gentamicin (25mg/L) for the selection of vectors.

Rose leaves agro-infiltrations: GUS-expression was evaluated in different types of leaves of rose genotypes; Leaves with a green upper side and red lower side (type A), light green leaves with dark green prominent veins

and red edges (type B), complete light green young leaves (type C) and dark green old leaves (type D). These leaves were collected from selected genotypes growing in different cultures and stored in humid conditions until infiltrations were done. For the preparation of bacterial suspension, overnight grown bacteria were processed by centrifugation (4500 rpm, 15 min at 22°C) and washed once in sterile water. The pelleted bacteria were re-suspended in sterile water or in infiltration buffer (Zottini *et al.*, 2008) additionally 0 and 100 µM of acetosyringone was added in *Agrobacterium* suspension when prepared in distilled water. The density of bacterial suspension was adjusted to OD₆₀₀: 0.5-2.0. Infiltrations were carried out as 2-10 spots in a detached rose leaf with a syringe (Yasmin & Debener, 2010). The leaves were stored at 16, 22 and 28°C in the dark for 3-days in a humid plastic box after infiltration and samples were collected on 4th day of treatment.

Evaluation of GUS expression through histo-chemical assay:

Twenty samples per condition for five experiments were collected and GUS histo-chemical assay was carried out (Jefferson *et al.*, 1987). Infiltration of staining solution is carried out by vacuum. The samples were left overnight at 37°C in staining solution and washed in 70% ethanol until chlorophyll was removed completely. *N. benthamiana* and rose leaves were infiltrated with water and used as controls.

Data collection and processing: Data for GUS expression levels was collected visually and classified as 0 to 3 on the basis of no GUS expression to the highest expression (Fig. 1). Kruskal-Wallis and Wilcoxon tests were used to evaluate data.

Results

For a successful transient expression assay in rose leaves different factors as host genotypes and their culturing methods, leaf age, bacterial density, additives to the bacterial suspension and temperature during incubation of infiltrated samples were optimized using GUS-intron reporter gene. First of all infiltration method was optimized in the leaves of rose genotype *Pariser Charme* (PC). In this regard, infiltrations with 1 ml needleless syringes was found as the best option to perform spot infiltrations in leaves, whereas vacuum infiltration (5 min at 200 mbar with 2-5 breaks) was not suitable for this assay. When vacuum infiltrated leaves were compared to syringe infiltrated leaves former showed very little or no GUS expression at all tested densities of bacteria. These observations about GUS expression were consistent in all rose genotypes selected for this study. Hence, syringes based infiltrations were carried out to optimize the assay.

Interaction of host genotypes and *Agrobacterium*:

Three rose genotypes (*Pariser Charme*, 91/100-5 and 88/124-46) were used as host of *Agrobacterium*. The host genetic background significantly influenced the agro-infections. Kruskal Wallis Test resulted in $p = 1.3 \times 10^{-19}$ that

means rose genotypes are effecting GUS expression in a highly significant manner or the ability of agro-infection. Two out of three rose genotypes, *Pariser Charme* and 91/100-5, allowed agro-infection and resulted in good GUS expression (Fig. 2). In contrast one genotype (88/124-46) responded as resistant to agro-infection. The obtained data for host genotype screening was monitored visually and rated as described in Figure 1.

Influence of host pre-culturing: The GUS expression was optimized in three rose genotypes growing in three different semi-controlled conditions (greenhouse, under plastic tunnels and *in vitro*). These pre-culturing conditions affected GUS transient expression significantly (Kruskal Wallis Test $p = 6.37 \times 10^{-16}$). *Pariser Charme* and 91/100-5 genotypes grew under tunnels with high humidity and temperature (above 30°C) displayed the highest GUS expression (Fig. 1). Genotype 88/124-46 never showed any GUS expression.

Influence of leaves age: GUS-expression was evaluated in the leaves of different physiological stages of three genotypes i.e. leaves with a green upper side and red lower side (type A), light green leaves with dark green prominent veins and red edges (type B), complete light green young leaves (type C) and dark green old leaves (type D). Although, the infiltration was very easy in old leaves, type B leaves of PC and type C leaves of 91/100-5 showed the highest GUS expression. Kruskal Wallis test ($p = 0.0008337$) revealed that the leaf age significantly affect the GUS expression.

Influence of additives: Infiltration medium as compared to distilled water had no effect on GUS expression. During this study autoclaved sterile distilled water was used as infiltration medium. In addition, acetosyringone was tested for its effect on inducing vir genes and GUS expression. Kruskal Wallis ($p = 0.172$) testing revealed that its effect on GUS expression is non-significant.

Influence of temperature during sample incubation:

The effect of four different temperatures 16°C, 22°C and 28°C was evaluated on transient GUS expression in infiltrated rose leaves. Temperature was found as an important parameter for this assay. It affected the expression of foreign gene considerably by influencing agro-infection in host (Kruskal Wallis $p = 4.1 \times 10^{-13}$). Temperatures 16°C and 28°C revealed significant differences in GUS expression when compare to the expression obtained at 22°C, the visual intensity of GUS coloration was seems to be higher at 22°C as compared to 16°C and 28°C (Figs. 3 & 4b). The GUS expression was not detected at 28°C and samples were dehydrated.

Influence of bacterial density: Expression was also affected by the density of bacteria that were infiltrated into the host tissues. Bacterial suspension having an OD₆₀₀ within a range of 0.5-1.0 did not show any GUS expression whereas higher densities 1.5- 2.0 resulted in good GUS expression level (Fig. 4b).

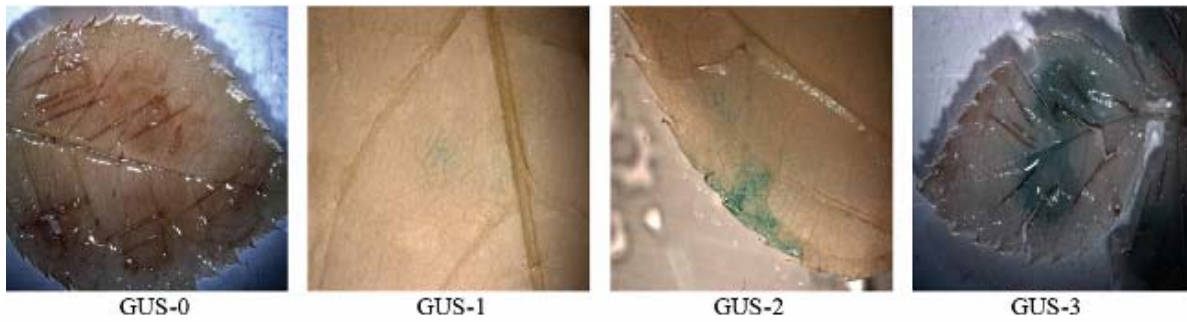


Fig. 1. Scoring for the histo-chemical GUS assay in rose leaves. The intensity of GUS expression is scored in numbers as indicated below the pictures.

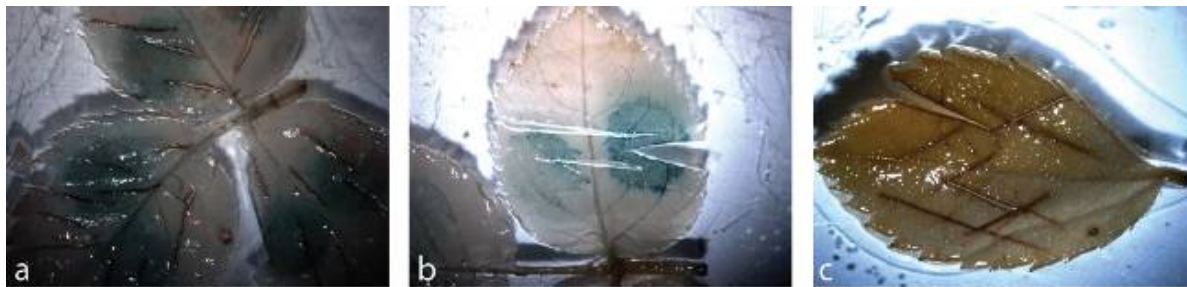


Fig. 2. Leaves of different rose genotypes showing GUS expression. These were infiltrated by GUS-Intron harboring *Agrobacterium* at $OD_{600} = 1.5$. a- *Pariser Charme*; b- 91/ 100-5; c- 88/ 124-46.

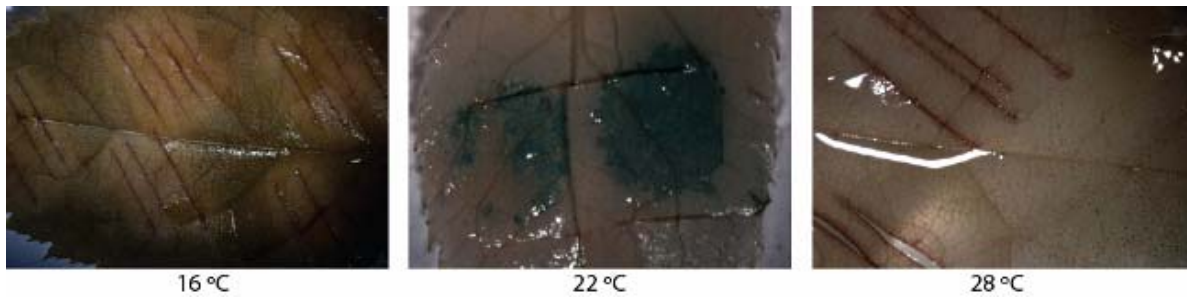


Fig. 3. Effect of temperature on GUS expression during sample incubations. Leaves of *Pariser Charme* were infiltrated by GUS-Intron harbouring *Agrobacterium* at $OD_{600} = 1.5$ and incubated at different temperatures (as indicated below the pictures). Samples were collected on 4th day post-infiltration and GUS histochemical assay was carried out.

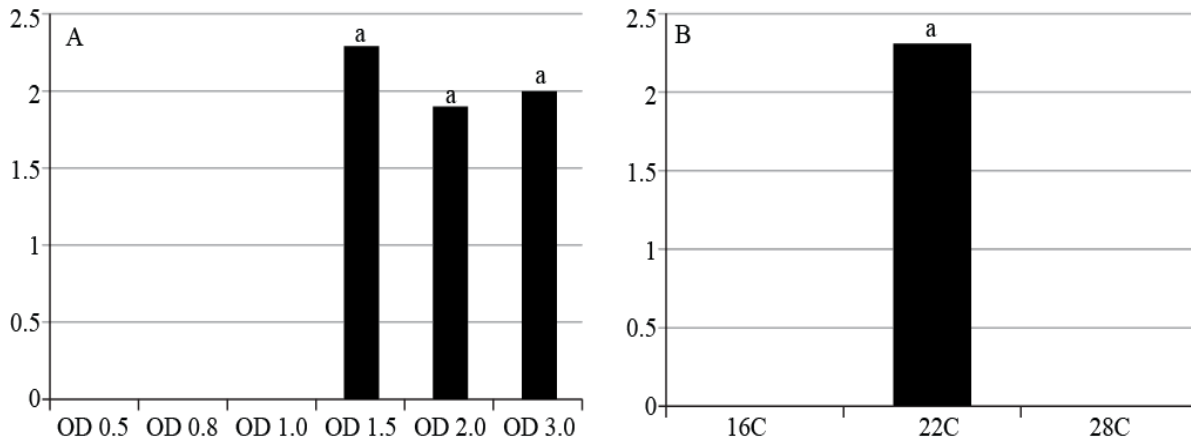


Fig. 4. Optimization of a successful *Agrobacterium* mediated transient GUS expression assay in the leaves of *Pariser Charme*. a- The effect of bacterial density; b- The effect of incubation temperature. The y-axis indicates the mean values of the GUS scores; the x-axes indicate the different treatments within each tested parameter. Different letters above each column indicate significant differences of the mean values at $p < 0.05$.

Table 1. Some important genes mapped in roses.

Gene	Function	References
<i>Blfo; d6</i>	Transition of stamens to petals, double flowers	Debener and Mattiesch (1999); Crespel <i>et al.</i> , (2002); Dugo <i>et al.</i> , (2005)
<i>Blfa</i>	Pink flower color	Debener and Mattiesch (1999)
<i>Rdr1</i>	Resistance to rose black spot	Von Malek <i>et al.</i> , (2000), Kaufmann <i>et al.</i> , (2003);
<i>Rdr3</i>		Whitaker <i>et al.</i> , (2010)
<i>Rpp1</i>	Resistance to rose powdery mildew	Linde <i>et al.</i> , (2004)
prickles	Prickles on the petioles	Rajapakse <i>et al.</i> , (2001)
<i>r4</i>	Recurrent blooming	Crespel <i>et al.</i> , (2002)

Discussion

The study presented here demonstrated rose leaves as a suitable system to carry out different kinds of transient expression studies for different genes of roses as characterizing promoters, transcription factors, investigating resident genes, protein trafficking, characterizing flower color or fragrance genes etc. The observations revealed the dependence of this assay on the host genotypes, age of leaves, host pre-culturing, infiltration method, bacterial density and temperature. According to, Santos-Rosa *et al.*, (2008) genetic background of a plant has significant effect on the transient gene expression. In present investigation the leaves of two genotypes (*Pariser Charme* and *91/100-5*) out of three (88/124-46) were found susceptible to agro-infection whereas 88/124-46 never allowed agro-infection in all observation. It is likely that there are some genetic factors affecting agro-infections as discussed by Zipfel *et al.*, 2006 and Yasmin & Debener, 2010. The phenomenon of being recalcitrant to genetic transformation using *Agrobacterium* is not specific for plant transient expression assays but also common in stable plant transformations. Different plant species as rice, maize, legumes, cucurbits, *Pinus* species, tomato, *Arabidopsis* and grape differ in their susceptibility to agro-infection; in addition, even different cells, tissues and organs of a plant show different tendency towards agro-infection (reviewed by Gelvin, 2000). The involvement of a specific LRR receptor kinase is reported in *Arabidopsis* that make some species recalcitrant to agro-infection (Zipfel *et al.*, 2006). Tzfira & Citovsky (2006) have reviewed the key host proteins necessary for the initial *Agrobacterium*-host interaction and T-DNA import, chromatin targeting, uncoating and its integration to host genome in detail suggesting their over-expression to increase the transformation efficiency. The different pre-cultures of host plants were found to affect the foreign gene expression significantly in leaves. The fresh shoots of *Pariser Charme* and *91/100-5* on their cuttings growing in plastic tunnels with high humidity and high temperature displayed significantly higher GUS level compared to greenhouse or *In vitro* grown plants which showed almost no GUS expression. There are many reports demonstrating the pre-culture of host plants with high humidity improve the transient expression positively (Zottini *et al.*, 2008; Yasmin, 2011). However, climatic conditions of plastic tunnels were not the only factor for a successful transient expression assay in rose. The genetic background is the determining factor for a successful transient expression as in all subsequent treatments 88/124-46 leaves always found resistant to agro-infection. Additionally, there is a possibility that some key host proteins as described by Tzfira & Citovsky (2006) may be affected by the conditions in

tunnels i.e. high temperature and humidity that make *Pariser Charme* and *91/100-5* more susceptible to agro-infection. It is important to notify that *91/100-5* never showed any GUS expression when grown in greenhouse or *in vitro*; it only showed GUS expression when grown in tunnels.

In agreement to Wroblewski *et al.*, (2005) we also found quite high variation in the GUS expression levels. The physiological stage or age of rose leaves have significant effect on GUS expression. Type B leaves (light green leaves with dark green prominent veins and red edges) of *Pariser Charme* and type C leaves (complete light green young leaves) of *91/100-5* grown in tunnels revealed the highest GUS level. The concentration of *Agrobacterium* suspensions also affected GUS expression significantly. The GUS expression was only detected at Densities ≤ 1.5 ; no expression was detected at lower densities. In contrast, Wroblewski *et al.*, (2005) and Santos Rosa *et al.*, (2008) observed weak expression even at bacterial densities of OD₆₀₀ 0.1. This could be due to the differences of physiological and genetic makeup among plant species. It was demonstrated earlier that the use of different growth media, infiltration media and addition of chemical components as acetosyringone influence the gene expression positively (McIntosh *et al.*, 2004). Acetosyringone facilitates the transfer of T-DNA into the host chromatid by inducing bacterial virulence genes. According to McIntosh *et al.*, (2004) acetosyringone is necessary for the good gene expression. In contrast, our study showed that such chemical additions in media did not improve the transient expression in rose leaves system. Wroblewski *et al.*, (2005) also found the same results and demonstrated that the without using acetosyringone good gene expression is possible. However, we found that the temperature affects the transfer of T-DNA in plants during *Agrobacterium* mediated transient transformations, significantly. This is in agreement to earlier studies of Dillen *et al.* 1997 and Yasmin, 2011. Post agro-infiltration, the rose leaves were maintained at different temperatures i.e., 16°C, 22°C and 28°C for 3 days, observations revealed that the 22°C is optimum for GUS expression.

In summary the GUS (β -glucuronidase) gene was used to optimize the transient gene expression system in rose leaves aiming at its future use to characterize rose resistance genes for the assessment of their activity against different fungal pathogens.

Acknowledgments

We thank Mrs. Ingrid Robota, Technical Assistant, Institute of Genetics, University of Hannover, for assistance in maintaining *in vitro* rose cultures for this study.

References

- Ahmed, I., M. S. Khalid, M. A. Khan and M. Saleem. 2011. Morpho-physiological comparison of cut rose cultivars grown in two production systems. *Pak. J. Bot.*, 43(6): 2885-2890.
- Byrne, D.H. 2009. Rose structural genomics. In: *Genetics and genomics of rosaceae*. (Eds.): K.M. Folta and S.E. Gardiner. Springer Science Business Media, pp. 353-380.
- Chaudhry, Z., S. Abbas, A. Yasmin, H. Rashid, H. Ahmed and M. A. Anjum. 2010. Tissue culture studies in tomato (*Lycopersicon Esculentum*) var. MoneyMaker. *Pak. J. Bot.*, 42(1): 155-163.
- Dillen, W., J. De Clercq, J. Kapila, M. Zambre, M. Van Montagu and G. Angenon. 1997. Effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. *The Plant Journal*, 6: 1459-1462.
- Dohm, A., C. Ludwig, D. Schiling and T. Debener. 2002b. Transformation of roses with genes for antifungal proteins to reduce their susceptibility to fungal diseases. *Acta Horticulturae*, 572: 105-111.
- Dohm, A., C. Ludwig, D. Schiling, T. Debener. 2002a. Transformation of roses with genes for antifungal proteins to reduce their susceptibility to fungal diseases. *Acta Horticulturae*, 572: 27-33.
- Gelvin, B.S. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 51: 223-56.
- Janssen, B.J. and R.C. Gardner. 1989. Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. *Plant Mol. Biol.*, 14: 61-72.
- Jefferson, R.A., T.A. Kavanagh and M.V. Bevan. 1987. GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.*, 6: 3901-3907.
- Marchant, R., J.B. Power, J.A. Lucas and M.R. Davey. 1998a. Biolistic transformation of rose (*Rosa hybrida* L.). *Annals of Botany*, 81: 109-114.
- Marchant, R., M.R. Davey, J.A. Lucas, C.J. Lamb, R.A. Dixon and J.B. Power. 1998b. Expression of a chitinase transgene in rose (*Rosa hybrida* L.) reduces development of blackspot disease (*Diplocarpon rosae* Wolf). *Molecular Breeding*, 4: 187-194.
- McIntosh, K.B., J.L. Hulm, L.W. Young and P.C. Bonham-Smith. 2004. A rapid *Agrobacterium*-mediated *Arabidopsis thaliana* transient assay system. *Plant Mol Biol Report*, 22: 53-61.
- Naeem, M., I. Naveed, S. M. S. Naqvi, S. M. Saqlan and T. Mahmood. 2013. Standardization of tissue culture conditions and estimation of free scavenging activity in *Viola odorata* L. *Pak. J. Bot.*, 45(1): 197-202.
- Santos-Rosa, M., A. Poutaraud, D. Merdinoglu and P. Mestre. 2008. Development of a transient expression system in grapevine via agro-infiltration. *Plant Cell Rep.*, 27:1053-1063.
- Tzfira, T. and V. Citovsky. 2006. *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Curr. Opin. Biotechnol.*, 17: 147-154.
- Van Engelen, F.A., J.W. Molthoff, A.J. Conner, J.P. Nap, A. Pereira and W.J. Stiekema. 1995. pBINPLUS: an improved plant transformation vector based on pBIN19. *Transgenic Res.*, 4: 288-290.
- Wroblewski, T., A. Tomczak and R. Michelmore. 2005. Optimization of *Agrobacterium* mediated transient expression assays for lettuce, tomato and *Arabidopsis*. *Plant Biotech. J.*, 3: 259-273.
- Yasmin, A. 2011. Rose and Black spot. VDM Verlag Dr. Müller GmbH & Co. KG. (ISBN 9783639328073).
- Yasmin, A. and T. Debener. 2010. Transient gene expression in rose petals via *Agrobacterium* infiltration. *Plant Cell Tissue and Organ Culture*, 102(2): 245-250.
- Zipfel, C., G. Kunze, D. Chinchilla, A. Caniard, J.D.G. Jones, T. Boller and G. Felix. 2006. Perception of the bacterial PAMP *EF-Tu* by the receptor *EFR* restricts *Agrobacterium*-mediated transformation. *Cell*, 125: 749-760.
- Zottini, M., E. Barizza, A. Costa, E. Formentin, C. Ruberti, F. Carimi and F.L. Schiavo. 2008. Agroinfiltration of grapevine leaves for fast transient assays of gene expression and for long-term production of stable transformed cells. *Plant Cell Rep.*, 27: 845-853.

(Received for publication 12 December 2011)