Somatic embryogenesis, transformation with PSAG12-IPT gene for leaf senescence retardation, and characterization of SERK genes in miniature potted rose (Rosa hybrida "Linda")
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Hedayat Zakizadeh

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April 2008
Somatic embryogenesis, transformation with $P_{SAG12}$-IPT gene for leaf senescence retardation, and characterization of SERK genes in miniature potted rose (*Rosa hybrida* 'Linda')

Ph.D. Thesis

Hedayat Zakizadeh

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University of Copenhagen
Denmark

April 2008
This study is dedicated to

My family

and

My friends
Acknowledgments

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H. Zakizadeh                        April 2008                      Copenhagen University, Denmark
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>2,4- dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>2iP</td>
<td>N6- (delta 2-isopentenyl)-adenine</td>
</tr>
<tr>
<td>4-CPA</td>
<td>p-chlorophenoxyacetic acid (pCPA)</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>CODEHOP</td>
<td>consensus degenerate hybrid oligonucleotide primer</td>
</tr>
<tr>
<td>DADS</td>
<td>days after dark-stress</td>
</tr>
<tr>
<td>DAF</td>
<td>day after flowering</td>
</tr>
<tr>
<td>DHZ</td>
<td>dihydrozeatin (or DZ)</td>
</tr>
<tr>
<td>DZ</td>
<td>dihydrozeatin (or DHZ)</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>GA₃</td>
<td>gibberellic acid</td>
</tr>
<tr>
<td>IAA</td>
<td>indole -3- acetic acid</td>
</tr>
<tr>
<td>IBA</td>
<td>indole -3- butyric acid</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich-repeat</td>
</tr>
<tr>
<td>LZ</td>
<td>leucine zipper (or ZIP)</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>NAA</td>
<td>1-naphthylacetic acid</td>
</tr>
<tr>
<td>NCBI</td>
<td>national center for biotechnology information</td>
</tr>
<tr>
<td>NOA</td>
<td>2-naphthoxyacetic acid</td>
</tr>
<tr>
<td>pCPA</td>
<td>p-chlorophenoxyacetic acid (4-CPA)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGR</td>
<td>plant growth regulator</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SPP</td>
<td>Ser-Proline-Proline motif</td>
</tr>
<tr>
<td>TDZ</td>
<td>thidiazuron</td>
</tr>
<tr>
<td>ZIP</td>
<td>leucine zipper (or LZ)</td>
</tr>
<tr>
<td>ZMP</td>
<td>zeatin riboside 5’-monophosphate</td>
</tr>
<tr>
<td>ZR</td>
<td>zeatin riboside</td>
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## Genes / proteins (symbols for genes are the same as proteins but in italic)

<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAB</td>
<td>chlorophyll a/b binding protein</td>
</tr>
<tr>
<td>IPT</td>
<td>isopentenyl transferase</td>
</tr>
<tr>
<td>RLCK</td>
<td>receptor-like cytoplasmic kinase</td>
</tr>
<tr>
<td>RLK</td>
<td>receptor-like kinase</td>
</tr>
<tr>
<td>SAG</td>
<td>senescence associated gene</td>
</tr>
<tr>
<td>SARK</td>
<td>senescence associated receptor-like kinase</td>
</tr>
<tr>
<td>SDG</td>
<td>senescence down-regulated gene</td>
</tr>
<tr>
<td>SERK</td>
<td>somatic embryogenesis receptor-like kinase</td>
</tr>
<tr>
<td>SIRK</td>
<td>senescence-induced receptor-like kinase</td>
</tr>
<tr>
<td>SSU</td>
<td>Rubisco small subunit</td>
</tr>
</tbody>
</table>
Abstract

Leaf senescence is a major cause of early quality loss in many ornamental crops during production, postproduction handling, transport, and display life. In some miniature potted rose cultivars; leaf yellowing, flower, bud and leaf abscission can be major problems during marketing and in the consumer’s home. Leaves of potted roses yellow and abscise under low light and high temperature conditions. Cytokinin can reduce leaf senescence in many species, and therefore, several plants have been transformed with a cytokinin biosynthesis gene (ipt) under the control of the senescence-specific (SAG12) promoter (P_{SAG12}-IPT). In the present study, this gene construct was used in Agrobacterium-mediated transformation of the miniature potted rose Rosa hybrida ‘Linda’ for reducing leaf senescence.

As somatic embryos are the most recommended explants for transformation of rose, regeneration via somatic embryogenesis was established in the miniature rose cultivar ‘Linda’ (Paper 1). Initially, in vitro cultures of eight miniature rose cultivars were established and then sufficient amounts of callus were produced using leaf explants of these cultivars on MS-media containing high levels of 2,4-D. Embryogenic callus was induced on calluses of six cultivars incubated on media containing either zeatin or TDZ. Although the frequency of somatic embryogenesis in ‘Linda’ was only 3.3%, further work on transformation was continued with this cultivar, because the promising results related to plant regeneration was obtained only in this cultivar. ABA was used for proliferation of embryogenic callus, and high levels of BAP together with low levels of NAA and GA₃ were used for shoot regeneration and elongation. The observed high demand of auxin for callus induction and of BAP for shoot regeneration appears to be due to low endogenous hormonal levels in miniature roses.

Among different A. tumefaciens strains used for transformation, transgenic shoots were obtained from explants treated with the AGL1 strain. The highest transformation frequency (10%) was obtained when ½ MS medium was used for the dilution of overnight culture of Agrobacterium. PCR and Southern blot analysis confirmed the existence of four transgenic lines with 1-6 copies of the nptII gene. The transformed lines did not show any morphological abnormalities at in vitro stage. However, root induction on shoots of these lines was more difficult compared to control shoots. In preliminary experiments, in response to dark-stress, transgenic plants showed better postharvest performance than control plants. Further analyses of the greenhouse-grown plants are
required to confirm these results before the transgenic lines can be used in breeding programs.

In order to find a reliable marker for recognizing the competent embryogenic callus from non-embryogenic callus of rose, four partial gene sequences belonging to the RLK super family were isolated (Paper 2). These genes were called *RhSERK1-RhSERK4* (Acc. No. EF631967-70) since they show high identity with genes from somatic embryogenesis receptor-like kinase (SERK) family. Expression analysis showed that the four isolated *RhSERK* genes were expressed in both embryogenic and non-embryogenic tissues, suggesting that they have a broader role in plant growth and development and cannot be considered reliable markers for recognizing embryogenic callus in the rose callus culture.

Finally, in the present thesis the relevant literature is discussed in relation to the experimental results of the study. To make the findings usable for breeding programs, more research is needed. Moreover, new molecular methods should be employed to find a reliable molecular marker for recognizing competent embryogenic callus at the early stage of culturing. Finally, the importance of *P_SAG12*-IPT transgenic roses in postharvest industry is discussed, as well as the perspectives of new selection methods with respect to the present GMO legislation in the world.
Dansk resumé


Forskellige *A. tumefaciens* stammer blev brugt til transformation, men alle transgene skud blev isoleret fra AGL1 behandlede eksplantater. Den højeste transformations hyppighed (10%) blev opnået, når overnatskulturen af *Agrobacterium* blev fornyet i ½ MS medie. Fire forskellige transgene linier blev isoleret, og 1-6 kopier af *nptII* genet blev verificeret i planterne ved hjælp af PCR og Southern blot. De transformerede linier viste ingen afvigende morfologiske træk på *in vitro* stadiet, dog var induktion af rødder og skud vanskeligere i disse linier sammenlignet med vildtype planter. I indledende eksperimenter, hvor planterne udsattes for mørkestress, havde de transgene planter en bedre holdbarhed end kontrol planter, men før de transgene linier kan indgå i forædling, er det dog nødvendigt at understøtte disse resultater med yderligere analyser af drivhus dyrkede planter.
For at finde en egnet markør til at skelne mellem embryogent og ikke-embryogent kallus, blev fire partielle gen sekvenser tilhørende RLK superfamilien isoleret (artikel nr. 2). Da disse gener har stor homologi til gener tilhørende familien af somatisk embryogenese receptor-lignende kinaser (SERK), blev de navngivet \textit{RhSERK1-RhSERK4} (Acc. No. EF631967-70). Ekspressionsanalyse af de fire isolerede \textit{RhSERK} gener viste, at de er udtrykt i både embryogent og ikke-embryogent kallusvæv, og derfor ikke kan bruges som markører for embryogenese hos roser, da de hos denne art sandsynligvis spiller en kompleks rolle i vækst og differentiering af kallus.

Denne afhandling indeholder desuden en diskussion af den relevante litteratur i relation til projektets eksperimentelle data. Yderligere undersøgelser er dog stadig nødvendige, for at gøre resultaterne anvendelige i forædlings øjemed. Desuden bør nyere metoder tages i brug for at finde en god molekyler markør til at genkende embryogent kallus på et tidligt stadie af vævskulturen. Til slut diskuteres betydningen af \textit{P\textsubscript{SAG12}-IPT} transgene roser for industrien, og der perspektiveres over nye selektionsmetoder i forhold til den nuværende GMO lovgivning.
Introduction
1. Introduction

The genus *Rosa* is the most important ornamental plant in the world, and numerous species and cultivars are produced widely for use as garden plants, cut flowers, potted plants and for the perfume industry (Horn 1992; Short and Roberts 1991). Among them, miniature potted roses are of great importance in Europe and their popularity is increasing in North America (Borch et al. 1995; Müller et al. 2001). In Europe with total production of 75 million pots in 2006 (M. Serek, personal communication), Denmark with around 41.1 million pots (Rasmussen 2007) and the Netherlands with around 24.0 million pots (T. Proll, personal communication), are the most important countries for the production of this attractive plant. Other major European countries for potted rose production are France, Germany and Italy (Pemberton et al. 2003). After Europe, North America, mostly Canada, with a production of around 25 million pots in 2006 has the highest miniature potted rose production (M. Serek, personal communication). The increased production requires transportation to new markets in other countries. Therefore, extensive efforts should be taken to improve the production and postproduction quality of these valuable plants (Serek and Andersen 1993).

In the production, and particularly during postproduction handling, transport, and display of many crops, mainly vegetables and ornamental plants, a major cause of early quality loss is leaf senescence (Buchanan-Wollaston 2003; Grierson 1987; Kader 1985; Lipton 1987; Mayak 1987; Schroeder et al. 2001). This process has also been seen in roses. Leaf yellowing, flower, bud and leaf abscission can be major problems during marketing of miniature potted roses (Clark et al. 1991; Müller et al. 1998; Serek and Andersen 1993). Under high temperature and low light conditions, the leaves of potted roses yellow and abscise and flower buds fail to open (Tjosvold et al. 1994). However, in miniature potted roses, most of the studies have focused on bud and flower senescence (Müller et al. 2000; Serek and Andersen 1993). There are only few reports on retardation of leaf senescence in miniature rose cultivars (Clark et al. 1991; Tjosvold et al. 1994).

At optimum conditions, when the leaf reaches a certain age, the senescence process will start. However, leaf senescence can be triggered by several internal and external factors including plant growth regulators, reproduction, aging, shading, light, temperature, water and nutrient availability, stress, pathogen attack, UV-B, ozone and wounding (Buchanan-Wollaston 2003; Hopkins et al. 2007; John et al. 2001; Lim et al. 2007; Miller et al. 1999; Smart 1994; Thomas and Stoddart 1980). Among plant growth regulators, ethylene and cytokinin have the most important role in senescence studies.
Introduction

(Noh et al. 2004). Effects of ethylene as an accelerator of senescence processes have been studied extensively (Mattoo and Handa 2004; Müller et al. 1998). Ethylene biosynthesis can be triggered by some environmental stresses. Most studies with the aim of senescence retardation have tried to either reduce ethylene production or making plants insensitive to ethylene (Müller et al. 1998; Noh et al. 2004; Serek and Andersen 1993).

Cytokinins have effects on many plant developmental processes including leaf senescence (Noh et al. 2004; Taiz and Zeiger 2002). Several experiments have shown that cytokinins are the most important senescence-retarding growth regulators (Buchanan-Wollaston 1997; Gan and Amasino 1997; Gan and Amasino 1996; Lim et al. 2007; Nam 1997; Noodén 2004; Taiz and Zeiger 2002). It has been demonstrated that senescence is accompanied by a decline in leaf content of cytokinin, and it has also been shown that spraying with artificial or natural cytokinin prevents leaf yellowing in some plants (Gan and Amasino 1997; Gan and Amasino 1996; Lim et al. 2003; Weaver et al. 1998). In miniature potted rose, spraying with cytokinins has reduced leaf yellowing (Clark et al. 1991; Tjosvold et al., 1994) and increased flower longevity (Serek and Andersen 1993). Therefore, producing a transgenic plant that over-produces cytokinin during the onset of senescence might solve this problem.

The enzyme that participates in the first and rate limiting step of cytokinin biosynthesis pathway is isopentenyl transferase (IPT) which is responsible for the transfer of the isopentenyl side chain to the adenine moiety. A gene encoding IPT has been well characterized in Agrobacterium tumefaciens where it forms part of the T-DNA (Gan and Amasino 1996; Kakimoto 2003). The effectiveness of the ipt gene in stimulating cytokinin biosynthesis has been demonstrated in several plant species under control of different promoters (Chapter 3, paragraph 3.4.3). Constitutive expression of ipt gene after induction of the promoter (such as CaMV 35S) leads to the overproduction of cytokinin which causes a range of effects attributed to cytokinins including physiological and morphological abnormalities. Therefore, the autoregulatory senescence-inhibition system was established using a suitable promoter to prevent cytokinin overproduction (Gan and Amasino 1995 and 1996). As reviewed by Gan and Amasino (1997) it has been shown that leaf senescence is associated with the up-regulation of many genes, termed Senescence Associated Genes (SAGs). One of these genes, which is expressed only during the onset of senescence is called SAG12 (Lohman et al. 1994). SAG12 is highly senescence-specific and is expressed in an age-dependent manner (Gan and Amasino 1997). Therefore, its promoter should be the best for driving the ipt gene at the onset of senescence. Gan and Amasino (1995) fused SAG12 promoter from Arabidopsis to the ipt
gene from *A. tumefaciens* and transformed tobacco plants with this construct (P$_{\text{SAG12}}$-IPT). Transgenic plants showed significant delay in leaf senescence, but there were no further abnormalities in plant growth and development compared with wild-type plants (Gan and Amasino 1995). The reason is that the increased levels of cytokinins reduce leaf senescence and attenuate the expression of the *ipt* gene and consequently prevent cytokinin overproduction (Gan and Amasino 1995). This construct has been transferred successfully to several plant species (Chapter 3, paragraph 3.4.3.2) including rose (Manuscript). Chapter three of the present thesis reviews leaf senescence, effects of cytokinins on this process, cytokinin biosynthesis pathways and genetic manipulation of leaf senescence using autoregulatory senescence-inhibition system in selected species.

Transformation of rose with different constructs using both biolistic methods and *Agrobacterium* strains has been reported previously (Condliffe et al. 2003; Derks et al. 1995; Dohm et al. 2001; Firoozabady et al. 1994; Kim et al. 2004; Li et al. 2002; Marchant et al. 1998; Van der Salm et al. 1997). Several factors are affecting transformation efficiency including cultivar, explant type, *Agrobacterium* strain, inoculation time and incubation period. Among different explants, embryogenic callus and somatic embryos have been shown to be the best materials for rose transformation (Condliffe et al. 2003; Firoozabady et al. 1994; Rout et al. 1991; Van der Salm et al. 1996). Regeneration efficiency after transformation was also dependent on several factors including types and concentrations of antibiotics (Li et al. 2002). After obtaining putative transgenic plants, analyses of the transformants are conducted using different methods such as PCR, RT-PCR, ELISA test and Southern blotting. In the present study, the construct P$_{\text{SAG12}}$-IPT was received from Prof. R. M. Amasino (Wisconsin University, Madison) and miniature potted rose (*Rosa hybrida* cv. Linda) was transformed with this construct (Manuscript). Chapter four of the present thesis will discuss different methods of transformation and various factors affecting the transformation efficiency with focus on transformation of rose cultivars, and the manuscript reports *Agrobacterium*-mediated transformation of the selected miniature rose cultivar with P$_{\text{SAG12}}$-IPT construct conducted in the present investigation.

Somatic embryogenesis is a process whereby somatic plant cells develop into entire plants via a series of stages similar to zygotic embryo development (Wann 1989). This phenomenon which first was recognized in carrots, has been shown in many plant species including species from the Rosaceae family (Raemakers et al. 1995; Wann 1989). Somatic embryos can be used for several purposes including genetic transformation. Chapter two discusses different stages of somatic embryogenesis and the role of different
factors in this phenomenon with more focus on rose cultivars. Paper one reports the development of a protocol for somatic embryogenesis of several miniature potted rose cultivars used for the present study.

Somatic embryogenesis is a genotype dependent and also time consuming process. For example, regeneration of whole plants via somatic embryogenesis in rose cultivars in the present study took almost two years. Therefore, several studies have been done to find a suitable marker for distinguishing the cells, which are competent to form somatic embryos from non-embryogenic cells. During somatic embryogenesis, alteration in expression of many genes occurs in induced cells (reviewed by Chugh and Khurana 2002) and among them, there is only one gene family, which is expressed in early embryogenesis in competent cells (Feher et al. 2003). This gene family has been termed somatic embryogenesis receptor-like kinase (SERK) and has been isolated from several species (Chapter 5, paragraph 5.3.2) including rose (Zakizadeh et al. 2007, paper 2). SERK genes have been used successfully as markers in carrot, *Arabidopsis* and *Dactylis glomerata* (Hecht et al. 2001; Schmidt et al. 1997; Somleva et al. 2000). Chapter five will give more information about this gene family and its expression patterns during somatic embryogenesis; as well as use of this gene as a marker in different species and isolation and characterization of SERK gene in rose.

This background leads to the following hypotheses for the present study:

1. Regeneration of miniature potted rose cultivars can be developed using protocols established for other rose cultivars.
2. Transformation of rose somatic embryos with the P$_{SAG12}$-IPT gene can be achieved using *Agrobacterium tumefaciens* strains.
3. Transgenic plants will exhibit significant delay in leaf senescence and will be, except for this characteristic, undistinguishable from control plants.
4. Homologue(s) of somatic embryogenesis receptor-like kinase (SERK) gene(s) exist in rose species.
5. SERK gene(s) from rose can be used as a marker for distinguishing cells which are competent to form somatic embryos in *in vitro* culture.
Literature Review
2. Somatic embryogenesis in genus *Rosa*

The genus *Rosa* has been propagated with different methods including cutting, layering, budding, grafting, using seeds and *in vitro* micro propagation (Horn 1992; Pati et al. 2006; Short and Roberts 1991). However, since the production of rose plants is driven by grower and customer demands for new traits such as pest- and disease resistance, longer vase life, new flower form, color and scent, new cultivars are produced via breeding programs. Since conventional breeding methods for introducing new genes into rose cultivars encounter some difficulties, new biotechnological methods could provide an easy way to introduce a desired gene into the gene pool (see Chapter 4). However, the genetic transformation of plants relies on several factors (see Chapter 4) including the occurrence of successful transformation event at the cellular level and successful plant regeneration from the transformed cells (Hansen and Wright 1999; Robinson and Firoozabady 1993). In general, *in vitro* regeneration is obtained via one of three ways: bud regeneration (from isolated meristems, apical or axillary buds in nodal segments), adventitious regeneration of organs (organogenesis) and somatic embryogenesis (Hansen and Wright 1999; Pati et al. 2006; Schum and Dohm 2003). Although, all these patterns have been reported in roses (see below), several rose transformation studies have shown that embryogenic callus and somatic embryos are the best materials for transformation of this plant species (see Chapter 4). In some cases, stem slices were used as explants for transformation (Van der Salm et al. 1997), but the final plant regeneration occurred via somatic embryogenesis (see Chapter 4).

Since the regeneration via somatic embryogenesis was used in the present study, I will focus more on this event in rose cultivars. However, regeneration via organogenesis in rose will be briefly discussed.

2.1 Regeneration via somatic embryogenesis

Somatic embryogenesis has been reported in more than 200 gymnosperm and angiosperm species including species from Rosaceae family (Raemakers et al. 1995). Somatic embryogenesis is a phenomenon whereby somatic plant cells undergo embryogenesis as an adaptation mechanism under certain conditions such as stress or application of plant growth regulators (PGRs) especially auxins and cytokinins (Feher et al. 2003). Even though they are not exactly the same as zygotic embryos (Dodeman et al. 1997), there are several reports on regeneration of normal plants from somatic embryos in different species including genus *Rosa* (paragraph 2.2). Somatic embryos can be used for
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Somatic embryogenesis is distinguished from organogenesis as having a unicellular rather than a multicellular nature (Wann 1989). In most cases, somatic embryos are produced from epidermal or subepidermal cells, either from single cells and/or multiple cells (Raemakers et al. 1995). To distinguish somatic embryos from embryos produced by gamete fusion, the term embryoid has been adopted by some researchers (Raghavan 1997; Wann 1989). However, this term has not been widely used. Therefore, the term somatic embryo is widely used today and is used in the present study.

2.2 Somatic embryogenesis in rose cultivars

Somatic embryogenesis in rose has been reported for the first time in cut rose cultivars, *R. hybrida* ‘Domingo’ and ‘Vickey Brown’ (De Wit et al. 1990). Frequency of somatic embryogenesis was between 0 and 7.1%, and phenotypically true to type plants were recovered (De Wit et al. 1990). Since then, several studies have been carried out on somatic embryogenesis induction in different rose cultivars. Embryogenic callus was induced in hybrid tea rose, *R. hybrida* ‘Royalty’ (Noriega and Söndahl 1991), potted rose, *R. hybrida* ‘Landora’ (Rout et al., 1991), garden rose, *R. rugosa* (Kunitake et al. 1993), dwarf miniature rose, *R. hybrida* ‘Meirutral’ (Arene et al., 1993) and different cut rose cultivars, *R. hybrida* ‘Sonia’, ‘Presto’, ‘Landora’, ‘Melody’, ‘Tineke’ and ‘White weekend’ (Derks et al. 1995). Likewise, embryogenic callus was induced from Floribunda rose, *R. hybrida* ‘Trumpter’ and ‘Glad Tiding’ (Marchant et al. 1996), rootstock cultivar, *R. hybrida* ‘Moneyway’ (Van der Salm et al. 1996), cut rose cultivars,
R. hybrida ‘Arizona’ (Murali et al. 1996), ‘Carefree beauty’ (Hsia and Korban 1996; Li et al. 2002) and ‘Gran Gala’ (Li et al. 2002) and miniature potted-rose, R. chinensis minima ‘Red Sunblaze’ (Hsia and Korban 1996; Li et al. 2002) and ‘Babykatie’ (Hsia and Korban 1996). In addition, somatic embryogenesis was reported in garden rose cultivars, R. canina and R. hybrida ‘Carl Red’ (Visessuwan et al. 1997), ‘Soraya’ (Kintzios et al. 1999), ‘Pariser charm’ and ‘Heckenzauber’ (Dohm et al. 2001) and hybrid tea rose, R. hybrida ‘Kardinal’ (Kamo et al. 2005). Hybrid tea roses can usually be used as either garden rose or cut rose cultivars. Therefore, except for R. hybrida ‘Meirutral’ (Arene et al., 1993) and R. chinensis minima ‘Red Sunblaze’ and ‘Baby Katie’ (Hsia and Korban, 1996; Li et al. 2002) which belong to miniature roses, the rest of the investigated cultivars were either garden roses or cut roses or used as both. In the present study, morphologically true to type plants were recovered via somatic embryogenesis in miniature potted rose cultivar, R. hybrida ‘Linda’ (Zakizadeh et al. 2008, paper 1).

Secondary somatic embryogenesis, which is the phenomenon where new somatic embryos are produced from primary somatic embryos, is also reported in some rose cultivars (Hsia and Korban, 1996; Li et al., 2002; Rout et al. 1991; Zakizadeh et al. 2008, paper 1). However, this phenomenon will not be further discussed here.

2.3 Different stages in somatic embryogenesis

Since direct somatic embryogenesis has not been observed in rose cultivars, the process of somatic embryogenesis via callus induction (indirect) with focus on rose cultivars will be explained here. Direct somatic embryogenesis has been explained elsewhere (Raghavan 1997; Wann 1989). In some species, different stages can be distinguished during the whole process of somatic embryogenesis including callus induction, embryogenic callus proliferation, somatic embryo development (differentiation and maturation), somatic embryo germination, shoot elongation and plant development. However, in rose, the process of somatic embryogenesis is sometimes combined with organogenesis and therefore all these stages (especially embryo germination) cannot be observed (Schum and Dohm 2003). For example, adventitious shoot regeneration from matured somatic embryos (Dohm et al. 2001) and adventitious root regeneration from these shoots (De Wit et al. 1990; Dohm et al. 2001; Li et al. 2002; Visessuwan et al. 1997; Zakizadeh et al. 2008) have been seen in some rose cultivars. However, different stages of somatic embryogenesis are dissected in this chapter. In order to avoid complexity, only the names of PGRs, and not concentrations, are mentioned in each table.
2.3.1 Callus induction

As a definition, induction is the initiation of a developmental sequence (Wann 1989). Induction starts after dedifferentiation of explant cells. Differentiated cells must become redetermined by successive mitotic divisions under an appropriate stimulus (Wann 1989). Similar to organogenesis, somatic embryogenesis depends on different factors, which include genotype (paragraph 2.3), type and age of explants, preculture conditions and endogenous and exogenous (Table 2-1) levels of PGRs (Jimenez 2005; Schum and Dohm 2003). Depending on the genotype, the frequency of somatic embryogenesis in roses may vary from below 1 to almost 100% (Schum and Dohm 2003). However, in most cases the frequency is very low.

Different types of explants have been used for induction of embryogenic callus in rose cultivars. Theoretically all plant tissues can be used for somatic embryogenesis, however, highly differentiated cells especially those with secondary cell walls take longer time to dedifferentiate (Wann 1989). Different explants including in vitro grown leaves (De Wit et al. 1990; Visessuwan et al. 1997; Dohm et al. 2001; Zakizadeh et al. 2008, paper 1), immature leaves and internodal stem segments (Rout et al., 1991; Hsia and Korban 1996; Li et al. 2002), in vivo grown leaves (Kintzios et al. 1999), immature seeds (Kunitake et al. 1993), in vitro derived petioles and roots (Marchant et al. 1996), adventitious roots (Derks et al. 1995; Van der Salm et al. 1996), petals (Murali et al. 1996), filaments (Noriega and Söndah 1991) and different flower parts (Arene et al. 1993) have been used for somatic embryogenesis in rose cultivars in different studies.

Jimenez (2005) has reviewed the effect of various PGRs on different stages of somatic embryogenesis. In rose, different auxins and cytokinins have been used for embryogenic callus induction (Schum and Dohm 2003). The appropriate stimulus for starting redetermination of differentiated cells in indirect somatic embryogenesis is auxin. Auxin works as the mitogenic substance, which results in redetermination (Wann 1989). However, Raemakers et al. (1995) showed that the type of PGR, which is used for somatic embryogenesis, depends on the type of explant. Embryogenic callus induction from zygotic embryos and flower associated explants occur mostly on PGR-free or cytokinin supplemented media, while in case of vegetative explants, it occurs on auxin and auxin/cytokinin supplemented media (Raemakers et al. 1995). Auxin has historically been the class of growth regulators used to effect somatic embryogenesis and among them, 2,4- dichlorophenoxyacetic acid (2,4-D) has most frequently been used (Wann 1989). The growth regulator 2,4-D seems to act as an effective stressor, being one of the
triggers of embryogenic development (Jimenez 2005). After 2,4-D, 1-naphthylacetic acid (NAA), indole-3- acetic acid (IAA), indole-3- butyric acid (IBA), Picloram and Dicamba have been used most frequently in dicot species (Jimenez 2005; Raemakers et al. 1995). However, the callus produced with only auxin is usually compact and non-embryogenic and has to be subcultured on PGR-free or cytokinin containing medium for somatic embryogenesis (Schum and Dohm 2003).

Table 2-1. Different PGRs, which have been used for embryogenic callus induction in different rose cultivars, are shown here. However, in some studies these stages cannot be recognized separately.

<table>
<thead>
<tr>
<th>PGRs used for induction of:</th>
<th>Non-embryogenic callus</th>
<th>Embryogenic callus</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetin + NAA or NOA*</td>
<td>2,4-D + Zeatin</td>
<td>De Wit et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>NAA + BAP + GA&lt;sub&gt;3&lt;/sub&gt;</td>
<td>NAA + BAP (higher level)</td>
<td>Noriega and Söndahl (1991)</td>
<td></td>
</tr>
<tr>
<td>2,4-D + BAP (low level)</td>
<td>PGR-free</td>
<td>Rout et al. (1991)</td>
<td></td>
</tr>
<tr>
<td>2,4-D</td>
<td>PGR-free</td>
<td>Arene et al. (1993)</td>
<td></td>
</tr>
<tr>
<td>2,4-D</td>
<td>PGR-free</td>
<td>Kunitake et al. (1993)</td>
<td></td>
</tr>
<tr>
<td>Dicamba</td>
<td>2,4-D + 2iP**</td>
<td>Derks et al. (1995)</td>
<td></td>
</tr>
<tr>
<td>NAA (or 2,4-D or IBA)</td>
<td>TDZ + GA&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Marchant et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>PGR-free</td>
<td></td>
<td>Van der Salm et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>Kintzios et al. (1999)</td>
<td></td>
<td>Kintzios et al. (1999)</td>
<td></td>
</tr>
<tr>
<td>Li et al. (2002)</td>
<td></td>
<td>Dohm et al. (2001)</td>
<td></td>
</tr>
<tr>
<td>Kamo et al. (2005)</td>
<td></td>
<td>Hettinga et al. (2001)</td>
<td></td>
</tr>
<tr>
<td>Zakizadeh et al. (2008)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 2-naphthyloxyacetic acid
** N6- (delta 2-isopentenyl)-adenine
*** p-chlorophenoxyacetic acid (4-CPA)

Among cytokinins, 6-benzylaminopurine (BAP) was used most frequently and followed by kinetin, zeatin and thidiazuron (TDZ) for somatic embryogenesis of many dicot species (Jimenez 2005; Raemakers et al. 1995). In some studies, gibberellic acid (GA<sub>3</sub>) has also been used together with other PGRs in this stage (Table 2-1). In roses usually NAA or 2,4-D have been used as auxin and zeatin or TDZ as cytokinin (Schum and Dohm 2003). However, different studies have used different types of auxin and cytokinin alone or in combination (Table 2-1) for somatic embryogenesis in different rose...
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cultivars. Moreover, embryogenic callus was obtained on PGR-free media in \textit{R. rugosa} (Kunitake et al. 1993).

In woody species including rose, one of the noticeable features of embryogenesis is that non-embryogenic and embryogenic callus are different from each other in color, texture and morphology and the last one, cell morphology, is the most convincing marker for recognizing the embryogenic callus (Wann 1989). In general, non-embryogenic callus is more compact and is green in color, while embryogenic callus usually has friable texture and creamy or white color. However, differences might be also seen among embryogenic callus of different cultivars of one species.

Other factors which have a role in embryogenic callus induction are the composition of basic culture medium (Marchant et al. 1996), light intensity (Kintzios et al. 1999), type of sugar of the medium (Dohm et al. 2001; Hsia and Korban 1996; Kunitake et al. 1993), type of gelling agent (Dohm et al. 2001; Van der Salm et al. 1996) and other substances which are added to the culture such as L-proline (Marchant et al. 1996; Rout et al. 1991; Van der Salm et al. 1996). SH-based media (Schenk and Hildebrandt 1972) has been used for both induction and maintenance of embryogenic callus in some studies (Derks et al. 1995; Kamo et al. 2005; Marchant et al. 1996; Van der Salm et al. 1996). Use of SH-based media led to higher frequency of somatic embryogenesis than when MS-based media (Murashige and Skoog 1962) were used (Marchant et al. 1996). Formation of somatic embryos was higher at 50 µmol m$^{-2}$s$^{-1}$ rather than at 150 µmol m$^{-2}$s$^{-1}$ light intensity (Kintzios et al. 1999). Using gelrite instead of agar stimulated somatic embryogenesis in \textit{R. hybrida} ‘Moneyway’ (Van der Salm et al. 1996). Presence of L-proline was necessary for the induction but not for proliferation of somatic embryos in \textit{R. hybrida} ‘Landora’ (Rout et al. 1991). However, in \textit{R. hybrida} ‘Trumpeter’ and ‘Glad Tiding’ L-proline enhanced somatic embryogenesis when used in embryo proliferation medium while it increased number of abnormal somatic embryos when used in embryo maturation medium (Marchant et al. 1996). Among different sugars (sucrose, fructose, glucose, galactose, lactose, maltose, mannose, mannitol and sorbitol) used for somatic embryogenesis in \textit{R. rugosa}, galactose increased the growth rate of embryogenic callus but inhibited the formation of somatic embryos, while fructose increased the somatic embryo formation (Kunitake et al. 1993). Likewise, addition of glucose increased somatic embryogenesis in some rose cultivars (Dohm et al. 2001; Hsia and Korban 1996). However, addition of L-proline or replacing sucrose by fructose had no effect on somatic embryogenesis in other rose cultivars (Dohm et al. 2001; Kintzios et al. 1999).
2.3.2 Embryogenic callus proliferation

In general, the frequency of embryogenic callus induction is low, therefore, once the embryogenic callus is obtained, it should be proliferated to provide enough material for further work. One of the differences between indirect organogenesis and somatic embryogenesis is that in organogenesis, the callus phase can not be kept for too long time because it will lose the morphogenic capacity, while the embryogenic callus can be kept and propagated for several years without losing the embryogenic capacity (Schum and Dohm 2003; Wann 1989). For example, regenerative capacity of embryogenic callus was maintained for about 6 months (Hsia and Korban 1996; Kunitake et al. 1993), more than 12 months (Li et al. 2002), up to 16 months (Rout et al. 1991), more than 18 months (Marchant et al. 1996; Noriega and Söndahl 1991), more than three years (Dohm et al. 2001; Zakizadeh et al. 2008, paper 1) and for up to five years (Kamo et al. 2005). This phase is very important because it provides the main material for genetic transformation (see Chapter 4) or micropropagation. However, the disadvantage of this phase is the creation of somaclonal variation in embryogenic callus, which can result in several phenotypic and genotypic abnormalities later in regenerated plants.

Table 2-2. Different PGRs, which have been used for proliferation and maintenance of embryogenic callus in some rose cultivars

<table>
<thead>
<tr>
<th>Different PGRs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D + Zeatin + GA₃</td>
<td>Noriega and Söndahl (1991)</td>
</tr>
<tr>
<td>NAA + BAP</td>
<td>Derks et al. (1995)</td>
</tr>
<tr>
<td>2,4-D (lower level than last stage)</td>
<td>Marchant et al. (1996)</td>
</tr>
<tr>
<td>NAA + Zeatin + GA₃</td>
<td>Dohm et al. (2001)</td>
</tr>
<tr>
<td>ABA (for S.S.E*)</td>
<td>Li et al. (2002)</td>
</tr>
<tr>
<td>2,4-D or (Dicamba + Kinetin)</td>
<td>Kamo et al. (2005)</td>
</tr>
<tr>
<td>ABA</td>
<td>Zakizadeh et al. (2008)</td>
</tr>
</tbody>
</table>

* Secondary somatic embryogenesis

The proliferation medium is either a new medium with combination of different PGRs (Table 2-2), a PGR-free medium (Visessuwan et al. 1997) or is the same medium, which has been used for callus induction (Hsia and Korban 1996; Kintzios et al. 1999; Kunitake et al. 1993; Marchant et al. 1996; Murali et al. 1996; Rout et al. 1991). In the later case, however, some elements of the media might be changed for enhancing the somatic embryogenesis such as using fructose or sucrose instead of galactose in R. rugosa (Kunitake et al. 1993). In general, composition of this media can affect the frequency of
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Further maturation and germination of somatic embryos. Kamo et al. (2005) showed that using SH-based media supplemented with 2,4-D in this stage would increase further maturation and decrease the germination, while using MS-based media supplemented with Dicamba and Kinetin will decrease the maturation and increase the germination of somatic embryos. Moreover, according to the same author, exposure to 2,4-D for a long period of time can inhibit plant regeneration and result in phenotypically abnormal plants (Kamo et al. 2005).

2.3.3 Somatic embryo development

This stage includes differentiation and maturation, and it mostly depends on the PGRs used in the previous stage. For example, in case of using abscisic acid (ABA) in proliferation stage, the somatic embryos in the embryogenic callus will be developed and get matured during the proliferation. Somatic embryos in embryogenic callus often exhibit a wide range of development within a culture. Therefore, highly developed embryos must be removed from the culture continually (Wann 1989). A result of insufficient maturation is the development of malformed somatic embryos and/or formation of fleshy leaves with fasciated (flattened) stems. This phenomenon is called precocious germination (Jimenez 2005; Raemakers et al. 1995). Different PGRs alone or in combination have been used at this stage for differentiation and maturation of somatic embryos in different rose cultivars (Table 2-3).

Table 2-3. Different PGRs, which have been used for differentiation and maturation of somatic embryos in some studies, are mentioned here. The hormonal combination for each stage has not been mentioned in the publications.

<table>
<thead>
<tr>
<th>PGRs for:</th>
<th>Differentiation</th>
<th>Maturation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Differentiation</strong></td>
<td><strong>Maturation</strong></td>
<td><strong>References</strong></td>
<td></td>
</tr>
<tr>
<td>GA&lt;sub&gt;3&lt;/sub&gt; + ABA (high level)</td>
<td>GA&lt;sub&gt;3&lt;/sub&gt; + lower ABA</td>
<td>Noriega and Söndahl (1991)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GA&lt;sub&gt;3&lt;/sub&gt; + ABA + 2,4-D</td>
<td>Marchant et al. (1996)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABA</td>
<td>Murali et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>Same medium as S.E induction</td>
<td>BAP + IAA</td>
<td>Kintzios et al. (1999)</td>
<td></td>
</tr>
<tr>
<td>ABA</td>
<td>ABA</td>
<td>Zakizadeh et al. (2008)</td>
<td></td>
</tr>
</tbody>
</table>

Maturation of somatic embryos was improved by transferring them into ABA supplemented media in some species (Raemakers et al. 1995). In addition to maturation, ABA prevents precocious germination in some species (Jimenez 2005). In rose also ABA has been used for this purpose (Table 2-3). However, PGR-free medium was also used in some cultivars (Hsia and Korban 1996; Kamo et al. 2005; Kunitake et al. 1993). Changing the composition of the medium such as using high osmotic medium (Noriega and
Söndahl 1991; Visessuwan et al. 1997) or adding other substances such as coconut water (Noriega and Söndahl 1991; Visessuwan et al. 1997) or sorbitol, adenine and malt extract (Kunitake et al. 1993) have been also used to increase the development of somatic embryos. High light intensity was essential for embryo maturation and germination in *R. hybrida* ‘Soraya’ (Kintzios et al. 1999). Adding activated charcoal to the MS-based media in this stage reduced the maturation but increased the germination frequency of somatic embryos (Kamo et al. 2005).

### 2.3.4 Germination of somatic embryos or adventitious shoot regeneration

The conversion frequency, or ability of somatic embryos to germinate and develop into plants, is variable among genotypes (Wann 1989). Formation of abnormal embryos is one of the reasons for low conversion frequency. In some cases low conversion frequency has been compensated by high levels of proliferation (Wann 1989). Conversion frequency in roses has been different being e.g. 60% (De Wit et al. 1990), 57% (Noriega and Söndahl 1991) and 15.2% (Kamo et al. 2005).

**Table 2-4.** Different PGRs used for germination of somatic embryos and further shoot development in some rose cultivars.

<table>
<thead>
<tr>
<th>PGRs for:</th>
<th>Germination</th>
<th>Shoot development</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination</td>
<td>Shoot development</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>PGR-free</td>
<td>BAP + IAA</td>
<td>De Wit et al. (1990)</td>
<td></td>
</tr>
<tr>
<td>BAP + GA₃</td>
<td>BAP + IAA</td>
<td>Noriega and Söndahl (1991)</td>
<td></td>
</tr>
<tr>
<td>BAP + IBA</td>
<td>BAP (lower level)</td>
<td>Rout et al. (1991)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAP + IBA</td>
<td>Van der Salm et al. (1996)</td>
<td></td>
</tr>
<tr>
<td>NAA</td>
<td>NAA</td>
<td>Murali et al. (1996)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAP or TDZ</td>
<td>Visessuwan et al. (1997)</td>
<td></td>
</tr>
<tr>
<td>BAP + IAA</td>
<td></td>
<td>Kintzios et al. (1999)</td>
<td></td>
</tr>
<tr>
<td>BAP + IBA + GA₃</td>
<td>BAP + NAA + GA₃</td>
<td>Dohm et al. (2001)</td>
<td></td>
</tr>
<tr>
<td>ABA or BAP</td>
<td>BAP</td>
<td>Li et al. (2002)</td>
<td></td>
</tr>
<tr>
<td>ABA</td>
<td></td>
<td>Kamo et al. (2005)</td>
<td></td>
</tr>
<tr>
<td>BAP + NAA + GA₃</td>
<td>BAP + NAA + GA₃</td>
<td>Zakizadeh et al. (2008)</td>
<td></td>
</tr>
</tbody>
</table>

Germination of mature embryos of rose is enhanced by different treatments including use of different PGRs (Table 2-4), using PGR-free media (Derks et al. 1995; Noriega and Söndahl 1991), half-strength salts (Murali et al. 1996; Noriega and Söndahl 1991; Rout et al. 1991), different sugar such as maltose (Marchant et al. 1996), placing somatic embryos
at low temperature (Marchant et al. 1996; Rout et al. 1991), in the dark (Marchant et al. 1996), under high light intensity (Kintzios et al. 1999) or using other substances such as adenine sulphate (Rout et al. 1991) or phloroglucinol (Murali et al. 1996) in the germination media. Low temperature, however, did not increase germination in other rose cultivars (Dohm et al. 2001).

In many rose cultivars, the regeneration occurs either via adventitious shoot formation or via a mixture of both, formation of adventitious shoots and germination of somatic embryos, regeneration processes (Schum and Dohm 2003). In some species like *Cyclamen persicum*, organogenesis and somatic embryogenesis can occur concomitantly in the same culture (Wann 1989). High concentrations of cytokinin will increase the adventitious shoot regeneration as well as further development of germinated embryos into plantlets (Noriega and Söndahl 1991; Schum and Dohm 2003).

2.3.5 Rooting and development of plants

For root development in bipolar somatic embryos as well as for adventitious root induction in propagated shoots (paragraph 2.4), rooting media containing half-strength salts supplemented with one or two auxins alone or in combinations have been used (reviewed by Pati et al. 2006). For example media containing IAA (Noriega and Söndahl 1991), IBA (De Wit et al. 1990), IBA and NAA (Dohm et al. 2001), IBA and IAA (Zakizadeh et al. paper 1) or without PGR (Li et al. 2002; Visessuwan et al. 1997) have been used before transferring to the greenhouse. NAA, IAA and IBA have been used more than other PGRs (Pati et al. 2006). Rooted plants are then grown in greenhouse conditions until flowering and further analyses.

2.4 Organogenesis in rose cultivars

Regeneration via organogenesis has been achieved in many plant species including rose cultivars (reviewed by Horn 1992; Pati et al. 2006; Short and Roberts 1991). In this phenomenon, differentiated cells will first dedifferentiate. This is stimulated by changing the endogenous hormonal balance (by wounding) and changing the PGR balance of the media. Similar to somatic embryogenesis, organogenesis can occur either directly or indirectly. In direct organogenesis, dedifferentiated cells will redifferentiate and then produce a shoot or root meristem, while in indirect organogenesis a callus phase will take place between dedifferentiation and redifferentiation phases (Schum and Dohm 2003). In general, a cytokinin is used for direct organogenesis and a combination of an auxin and a cytokinin for indirect shoot organogenesis. Both direct and indirect organogenesis has
been reported in roses (Pati et al. 2006; Schum and Dohm 2003). In, most cases of direct organogenesis, TDZ has been used for induction and BAP for regeneration of shoots, while in indirect organogenesis BAP have been used mostly together with NAA (Pati et al. 2006). In direct shoot organogenesis, the formation of shoot initials has been observed from subepidermal cell layers in the region of the vascular bundles of petioles, leaf veins and pericycle of roots (Schum and Dohm 2003). In root organogenesis, which is called rhizogenesis, different auxins, either alone or in combinations (paragraph 2.3.5) were used in the medium. Other factors including genotype, age and size of explants, media, inorganic salts, carbohydrates, physical factors including light and temperature, preculture conditions and interacting endogenous phytohormone levels also have effects in organogenesis (Pati et al. 2006; Schum and Dohm 2003). Glucose was better than sucrose for shoot organogenesis in R. hybrida “Carefree Beauty”, however this effect was genotype dependent (Hsia and Korban 1996). In R. damascene deletion of ammonium nitrate from the MS media and addition of calcium ionophore enhanced the formation of adventitious buds on callus (Ishioka and Tanimoto 1990). In leaf explants of R. damascene, addition of AgNO₃ enhanced direct shoot organogenesis (Pati et al. 2004). Different explants including leaf and root segments have been used successfully for direct organogenesis (Lloyd et al. 1988; Pati et al. 2004). However, a wider range of explants was used for indirect organogenesis (Hsia and Korban 1996; Ishioka and Tanimoto 1990; Li et al. 2002; Lloyd et al. 1988).

2.5 Analysis of the regenerated plants

After obtaining the regenerated plants, either from somatic embryogenesis or from organogenesis, those plants have to be evaluated for genotypic and phenotypic characteristics. Somaclonal variation can occur during regeneration especially via indirect organogenesis and somatic embryogenesis. Plants are checked for different traits including plant architecture, growth habit, presence of prickles (thorns), leaf size, shape and number of leaflets, leaf variegation, petal number, form and color, flower filling and sterility (Arene et al. 1993; De Wit et al. 1990; Raemakers et al. 1995). For example, morphological abnormalities including thick and fleshy stems, abnormal leaves, high number of thorns, different petal numbers and shape, dwarf growth habit and different flower color have been reported in some rose cultivars (Arene et al. 1993; De Wit et al. 1990; Lloyd et al. 1988). Moreover, genotypic characteristics of regenerated plants have been analyzed using flow cytometry and AFLP analysis (Dohm et al. 2001; Van der Salm et al. 1996).
3. Manipulation of leaf senescence

Senescence causes marked economical losses in agricultural production of ornamental plants, fruits and vegetables (Buchanan-Wollaston 2003; Grierson 1987; Kader 1985; Lipton 1987; Mayak 1987; Pogson and Morris 2004; Schroeder et al. 2001). Senescence processes, which occur during growth and development, postharvest and especially handling of crops, are very important in developmental studies (Buchanan-Wollaston 2003). Several published reviews on senescence (Danon et al. 2000; Gan and Amasino 1996; Hopkins et al. 2007; Noodén 2004; Noodén et al. 1997; Paliyath and Droillard 1992; Thomas et al. 2003; Thompson et al. 1998) and especially on leaf senescence (Buchanan-Wollaston 1997 and 2003; Chandlee 2001; Lim et al. 2003 and 2007; Nam 1997; Smart 1994; Thomas and Stoddart 1980; Yoshida 2003) reveal the importance of this process for human life. Controlled atmosphere storages are costly and are usually used for harvested fruits and vegetables. The external application of plant growth regulators such as cytokinin, GA3, IAA and 2,4-D as well as other chemicals like ascorbic acid and EDTA for delaying senescence in some crops has been reported (Gan and Amasino 1996; McCabe et al. 2001). Another way for senescence retardation in order to reduce damages during this process as well as for obtaining increased postharvest storage and stress tolerance in agricultural products is to produce resistant cultivars with breeding methods. For genetic manipulation of senescence, a better understanding of molecular changes undergoing senescence is necessary (Noh et al. 2004; Noodén et al. 1997). Therefore, I will start with the definition of senescence and different factors that can induce or affect senescence. Then, I will discuss how this information could be used for manipulation of senescence.

3.1 Definition of senescence

Senescence is an age-dependent process, which normally occurs at the terminal phase of biological development of the life of the plant (Lim et al. 2007; Thompson et al. 1998). It can also be induced prematurely by environmental conditions (Buchanan-Wollaston 2003). It is not an accidental and passive, but a complex and highly regulated process, which occurs at various organization levels including individual cells, tissues (like in vessel elements), organs (leaf, floral and fruits senescence) and whole plant (monocarpic senescence) (Buchanan-Wollaston 1997; Taiz and Zeiger 2002; Thompson et al. 1998). Senescence at cellular level is called programmed cell death (PCD), which plays an important role in animal development and has been studied extensively (Danon et al. 2000). In plants, PCD occurs in differentiation of xylem tracheary elements.
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(xyleogenesis), formation of abscission zone, sex determination, in the formation of aerenchyma in waterlogged roots and also in response to pathogen attack by making localized cell death around the infection site which is called hypersensitive response (Buchanan-Wollaston 2003; Danon et al. 2000; Gan and Amasino 1996; Nam 1997). However, there is no general agreement about the boundaries and overlaps between ageing, senescence, hypersensitive response, death, lesions, ripening, postharvest deterioration, chlorosis, necrosis and so on (Thomas et al. 2003). The difference between senescence and developmental aging (or in brief, aging) is that in contrast to aging which is referring to all changes which occur with time, without reference to death, senescence always ends in death. Aging would determine when senescence starts (Lim et al. 2003; Smart 1994). More on definition and relationship of some of these terms including senescence, aging, apoptosis and PCD has been reviewed elsewhere (Noodén 2004; Thomas et al. 2003).

3.2 Leaf senescence and its benefits

As the aim of the current study is to manipulate leaf senescence, this chapter will focus more on this area. As a definition, leaf senescence is the sequence of biochemical and physiological events in the final stage of development from mature, fully expanded state until death (Smart 1994). Despite from its detrimental effects, it has some advantages to both human and plant life including its aesthetic value in landscape design and importance in nutrient relocation (Gan and Amasino 1997; Smart 1994). Therefore, it is a critical process for the fitness of plants and regarded as an evolutionarily acquired genetic process (Nam 1997). The color change is due to both preferential degradation of chlorophylls compared with carotenoids and to the synthesis of new compounds such as anthocyanins and phenolics (Smart 1994). Senescence of one part of a plant is often necessary for development of other parts of the plant (Hopkins et al. 2007). As new leaves are produced from the shoot apical meristem, the lower leaves are shaded and their efficiency for photosynthesis is reduced. Therefore, the plants recover their valuable resources like sugars, proteins and nutrients (such as N, P, K, Mo, Cu and Fe) by transporting them back to other organs like specialized cells in trunk (in deciduous trees) or developing seeds (in annual crops) via senescence process (Buchanan-Wollaston 2003; Taiz and Zeiger 2002). Remobilization of nutrients from leaves to storage tissues like seeds during senescence is slow and the cells must remain viable until finishing this process and after that death will occur (Hopkins et al. 2007). All these changes are the results of alterations in gene expression, which will be discussed later in this chapter.
3.3 Different factors affecting initiation of senescence

Different internal and external (environmental) factors affect the initiation and continuation of various types of senescence. Internal factors include plant growth regulators (PGRs), reproduction, developmental age and shading. Among them, plant growth regulators are of great importance and this will be explained in more details in this chapter.

External factors, which can prematurely induce senescence, include light, temperature, water and nutrient availability, stress, pathogen attack, UV-B, ozone, wounding and others (Buchanan-Wollaston 2003; Hopkins et al. 2007; John et al. 2001; Lim et al. 2007; Miller et al. 1999; Smart 1994; Thomas and Stoddart 1980). Figure 3-1 illustrates some of these factors with a regulatory pathway of leaf senescence (Lim et al. 2007). Some of these factors might induce some specific senescence genes and some might induce all senescence genes, but they seem to be coordinated through a common signaling pathway (Hopkins et al. 2007; Nam 1997).

Figure 3-1. Different internal and external factors triggering leaf senescence in plants (Lim et al. 2007).
3.3.1 Effects of PGRs on leaf senescence

The hormonal pathways appear to play in all stages of leaf senescence including initiation, progression and terminal phases of leaf senescence (Lim et al. 2007). Among PGRs, cytokinins, auxins and gibberellins are known to inhibit leaf senescence, while ethylene, ABA, brassinosteroides, jasmonates (JA) and salicylic acid are known to promote this process (Noodén 2004; Smart 1994). However, since each PGR affects various events in a complex manner, it causes difficulties to assay the role of hormonal pathways in leaf senescence (Lim et al. 2007). Effects of PGRs depend on their concentrations and conditions and the plant species (Gan and Amasino 1996).

Among PGRs, which inhibit leaf senescence, cytokinins are of great importance (Gan and Amasino 1996). This class of PGRs has been used commercially for reducing leaf yellowing with either exogenous application on leaves or making transgenic plants that overproduce cytokinins (Gan and Amasino 1997; Lim et al. 2007; Noh et al. 2004). Therefore, I will focus on different aspects of this class of hormones in this chapter.

3.3.1.1 Cytokinins and their role in leaf senescence

The plant hormone cytokinin was first identified as a factor that stimulates cell divisions in the presence of auxin (Carimi et al. 2003; Noh et al. 2004). It has been called kinetin and is an adenine (or aminopurine) derivative, 6-furfurylamidopyrimidine (Taiz and Zeiger 2002). Zeatin is the most prevalent natural cytokinin in higher plants and is available in both cis and trans forms of which the later one is more active (Kakimoto 2003). Other substituted aminopurines, which are active as cytokinins, are N6- (delta 2-isopentenyl)-adenine (2iP) and Dihydrozeatin (DZ or DHZ). Synthetic cytokinins include benzylaminopurine (BAP) and thidiazuron (TDZ). Usually zeatin is the most abundant naturally occurring free cytokinin, but DHZ and 2iP are also commonly found in higher plants and bacteria (Taiz and Zeiger 2002). The zones of cytokinin production in plants are root tips, shoot apical meristem, cambium and immature seeds and among them root tips are the major sites of production (Miyawaki et al. 2004). Root produced cytokinin is transferred into the xylem together with water and nutrients (Taiz and Zeiger 2002).

Cytokinins have effects on many physiological and developmental processes of plants including the control of apical dominance and leaf senescence, assimilate import and partitioning, nutrient mobilization, flowering time, regulating shoot apical meristem and root growth, vascular tissue differentiation, floral development, breaking of bud dormancy and seed germination (Jordi et al. 2000; Noh et al. 2004; Sun et al. 2003; Taiz
and Zeiger 2002). External application of cytokinin exhibits various effects such as shoot initiation from callus cultures, promotion of axillary bud growth, stimulation of pigment synthesis, inhibition of root growth and retardation of senescence (Gan and Amasino 1996; Medford et al. 1989; Tjosvold et al., 1994).

Several experiments have identified cytokinins as the most effective senescence-retarding growth regulators (Buchanan-Wollaston 1997; Gan and Amasino 1996; Lim et al. 2007; Nam 1997; Taiz and Zeiger 2002). As the main zone of cytokinin production is the root, all treatments, which decrease its biosynthesis such as de-rooting, salt stress, water logging or mineral deficiency will promote leaf senescence (Smart 1994). In the opposite way, increasing the endogenous cytokinin levels will delay leaf senescence (will be explained later). It is known that endogenous levels of cytokinins drop during leaf senescence (Gan and Amasino 1996; Lim et al. 2007). Therefore, external application of cytokinins should delay leaf senescence. Treatment of detached leaves of many species with cytokinins delayed leaf senescence processes while it had no effect on attached leaves (Gan and Amasino 1997; Gan and Amasino 1996; Lim et al. 2003; Weaver et al. 1998). However, attached leaves of *Nicotiana rustica* re-greed after treatment with cytokinin (Zavaleta-Mancera et al. 1999). Treatment with cytokinin has also increased the abundance of both proteins and corresponding transcript levels of both Rubisco and chlorophyll a/b binding (CAB) proteins in different tissues of variety of plant species (Gan and Amasino 1996). Moreover, spraying of cytokinins on some miniature potted rose cultivars reduced leaf yellowing (Clark et al. 1991; Tjosvold et al., 1994). Exogenous application of cytokinins also delayed leaf senescence in young detached *Arabidopsis* leaves in either light or darkness (Weaver et al. 1998). However, in detached *Arabidopsis* leaves, the effects of exogenous cytokinin depended on the levels of endogenous cytokinins of the leaf (Kudryakova et al. 2001). The *Arabidopsis* mutant with high endogenous levels of cytokinin exhibited low sensitivity to exogenous ethylene (Kudryakova et al. 2001). Cytokinin application also retarded senescence in *Petunia* corolla explants (Taverner et al. 1999). Cytokinins are generally less effective in preventing expression of senescence genes in older *Arabidopsis* leaves, which have already started senescence (Weaver et al. 1998). Therefore, it cannot totally prevent leaf senescence but has a strong effect. In contrast to young leaves, old leaves will not produce cytokinin or produce very low amounts and they are dependent on root-produced cytokinins (Lim et al. 2003). In cotyledons of sunflower (*Helianthus annuus*), cytokinin pre-treatment partially blocked the senescence-promoting effects of methyl jasmonate (Naik et al. 2002). Foliar application of cytokinin also delayed heat-induced leaf
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senescence or promoted heat tolerance of creeping bentgrass (*Agrostis stolonifera*) by maintaining higher chlorophyll content and photochemical efficiency (Veerasamy et al. 2007).

Expression of a senescence-specific gene, *SAG12* (paragraph 3.4.1.3), declined rapidly in detached *Arabidopsis* leaves treated with 0.1 mM BAP (Noh and Amasino 1999). Expression of *SARK* gene that has a role in regulation of leaf senescence was also delayed by addition of cytokinin to detached leaves of *Phaseolus vulgaris* in the dark (Hajouj et al. 2000). Therefore, there should be some genes that link the cytokinin response to leaf senescence (Lim et al. 2003). Despite their remarkable effects in delaying leaf senescence, how cytokinins affect this phenomenon is still unknown (Lim et al. 2007; Noh et al. 2004).

3.3.1.2 Role of other PGRs in leaf senescence

Among other PGRs, ethylene is a very important hormone and it plays a prominent role in senescence in some species (Kader 1985; Mattoo and Handa 2004; Nam 1997). It has long been known as an endogenous regulator of senescence, including fruit ripening, flower and leaf senescence (Woo et al. 2004). In *Petunia* corolla senescence, it has been shown that ethylene promotes senescence by inactivation of cytokinins (Taverner et al. 1999). Disruption of the ethylene signaling pathway delays the onset of leaf senescence, fruit ripening and flower senescence in some species (Mattoo and Handa 2004; Noh et al. 2004; Thomas et al. 2003). Expression of *SAG12* gene (paragraph 3.4.1.3) was not detected in young leaves after ethylene exposure but was expressed in old leaves (Weaver et al. 1998). This observation indicates that ethylene can only induce senescence after the leaves have reached a certain developmental age (Buchanan-Wollaston 2003; Lim et al. 2003).

Abscisic acid (ABA) is also known to induce senescence (Weaver et al. 1998). Expression of many senescence genes (except for *SAG12*) was up-regulated after treatment with ABA (Weaver et al. 1998). Studies on salicylic acids and brassinosteroids have shown that these hormones cause premature senescence symptoms (Lim et al. 2003). Jasmonates are also assumed to be potent senescence-promoting substances in some species (Naik et al. 2002). Auxins and GA3 have been reported to act as senescence-inhibitors, however, different concentrations of these hormones might effect differently. The further role of some of PGRs on senescence has been discussed
elsewhere (Buchanan-Wollaston 1997 and 2003; Lim et al. 2007; Nam 1997; Noodén 2004; Smart 1994; Taiz and Zeiger 2002).

3.4 Manipulation of senescence using cytokinin overproduction

Since different signaling pathways are involved in gene expression during senescence processes, it is not possible to block the whole process by mutation of one single gene (Buchanan-Wollaston 2003). As mentioned before, in contrast to necrosis, which occurs by physical damage, poisons or other external injury, senescence is a viable and highly regulated process and leaves remain viable and active until the end of this process. Therefore, senescence is a reversible process and can be manipulated. A completely yellow leaf, which has mobilized most of its nutrient content, can be induced to re-green by different treatments (Buchanan-Wollaston 2003; Gan and Amasino 1996). One of the most important inhibitors of senescence is the plant hormone cytokinin as explained above (paragraph 3.3.1.1). To exploit the effects of this hormone for retardation of senescence in plants, we either need to spray plants with cytokinin (which is a time consuming and costly process and also the efficiency of hormone uptake and transport must be considered which is not easy to measure) or making transgenic plants, which overproduce cytokinins (Gan and Amasino 1996). For making transgenic plants, we need to know the cytokinin biosynthesis pathway and essential genes participating in this process. Since high concentrations of cytokinin can either trigger the PCD via increasing ethylene biosynthesis (Carimi et al. 2003) or have disadvantages like abnormality in morphology of plants, biosynthesis of this hormone in transgenic plants has to be regulated using a suitable promoter. To select a suitable promoter, we need to know what is going on during senescence (Noodén et al. 1997). For this reason, I will preface this section with an attempt to explain the genetic regulation in plant tissues that undergoing senescence, then present biosynthesis of cytokinin, and finally I will talk about the autoregulatory cytokinin biosynthesis pathway.

3.4.1 Molecular regulation of leaf senescence

In the course of leaf senescence various physiological, morphological and biochemical changes occur at the cellular level, which are results of changes in gene expression. These changes include up-regulation of some genes and down-regulation of other ones, which are regulated by other factors involving light and nitrogen status of the plant. Among these genes some have more importance in leaf senescence, which will be discussed more in detail.
3.4.1.1 Cytological changes during leaf senescence

During leaf senescence cells undergo the sequential disorganization of cellular organelles (Nam 1997). Among organelles, which are destroyed during leaf senescence, chloroplasts are the first organelles that are broken down leading to decrease in chlorophyll (Gan and Amasino 1996 and 1997; Prochazkova and Wilhelmova 2007; Taiz and Zeiger 2002). Chloroplasts of senescing leaves show reduced volume, spherical shape and reduced thylakoid system (Prochazkova and Wilhelmova 2007). Chlorophyll is associated with thylakoid membranes, which are morphologically disorganized and finally destroyed following the onset of senescence (Thompson et al. 1998). Chloroplasts that contain up to 70% of the leaf protein and consequently a large portion of leaf nitrogen, typify a healthy leaf tissue (Gan and Amasino 1997; Thomas et al. 2003).

One of the first events in senescence is loss of selective permeability of membranes due to metabolism of membrane lipids, in particular phospholipid, and consequently a large decrease in lipid fluidity of the membrane. This leads to conformational changes in membrane key proteins such as ion pumps and consequently loss of membrane function (Hopkins et al. 2007; Thompson et al. 1998). The degradation of membrane lipids occurs by collaborative activity of different membranous lypolytic enzymes such as phospholipase D, phosphatidate phosphatase, lipoxygense and enzymes that cause alkane synthesis (Paliyath and Droillard 1992). The activity of some of these enzymes has been shown to be initiated by elevated concentrations of $Ca^{2+}$ in the cytosol. The release of $Ca^{2+}$ into the cytosol, however, might be linked to ethylene biosynthesis (Paliyath and Droillard 1992). Thylakoid lipid degradation in senescing leaves seems to be mediated by $\alpha$-galactosidase, $\beta$-galactosidase and galactolipase enzymes (Thompson et al. 1998). One of the major leaf proteins is Rubisco, which undergoes degradation by cysteine protease and a serine protease during leaf senescence (Nam 1997). However, since cells are viable and need energy during senescence processes, leakiness of mitochondria membranes does not happen until the last steps of senescence (Hopkins et al. 2007; Lim et al. 2003). Nuclear DNA also remains intact and works functionally until the last steps of this process, whereas total RNA levels fall rapidly with the progress of senescence (Buchanan-Wollaston 1997; Lim et al. 2003; Nooden et al. 1997). In the last stage of leaf senescence when the leaf is completely yellow, chromatin condensation and DNA laddering is observed (Yoshida 2003). The nucleic acids, especially rRNA, are used as an important source of carbon, nitrogen and phosphorus within a senescing cell (Prochazkova and Wilhelmova 2007). In addition to these senescence-associated enzymes, various hydrolytic enzymes such as proteases,
lipases, chlorophyllase, ribonucleases, cell wall hydrolases, carbohydrate and nitrogen metabolizing enzymes, and stress responsive proteins are also synthesized during senescence processes (Buchanan-Wollaston 2003; Hinderhofer and Zentgraf 2001; Hopkins et al. 2007; Nam 1997; Prochazkova and Wilhelmina 2007). Biosynthesis of these enzymes is the result of changes in expression of some senescence-specific genes (Gan and Amasino 1997; Gepstein et al. 2003).

3.4.1.2 Senescence-related genes (SAGs and SDGs)

Molecular studies comparing gene expression at different stages of leaf development from non-senescing leaves to late stages of senescence have shown changes in expression of many genes in *Arabidopsis* (Lohman et al. 1994). Similar experiments using microarrays have revealed changes in expression of more than 1400 genes during leaf development in *Arabidopsis* (Buchanan-Wollaston et al. 2003). Expression of many genes is up-regulated during senescence processes (Gepstein et al. 2003). These genes are designated as senescence-associated genes (SAGs) by Lohman et al. (1994), and often encoding proteins that are capable of implementing cell death (Gan and Amasino 1997; Hopkins et al. 2007; Nam 1997). Understanding the regulation and expression of SAGs, which have also called SEN (senescence-enhanced) genes is of great importance in the genetic manipulation of senescence (Buchanan-Wollaston 2003). A large number of SAGs has been isolated from various species (Chandlee 2001; Gan and Amasino 1997; Gepstein et al. 2003; Noh and Amasino 1999; Woo et al. 2004). In contrast to SAGs, expression of some genes is decreased during senescence processes (Fig. 3-2). These genes, which are called senescence down-regulated genes (SDGs), include many genes encoding metabolic enzymes, genes which are active in photosynthesis such as chlorophyll a/b binding protein (CAB) and genes encoding Rubisco small subunit, as well as genes which repress the senescence programs (Lohman et al. 1994; Nam 1997; Taiz and Zeiger 2002).

There are also some genes, which are involved in the degradation of senescence regulatory factors (Lim et al. 2003). All senescence genes are regulated by several transcription factors like members of NAC and WRKY families (Hinderhofer and Zentgraf 2001; Hopkins et al. 2007; Yoshida 2003). The *AtWRKY53* gene was expressed at a very early stage of leaf senescence before the expression of *SAG12* became detectable, but decreased during the later stages of senescence, which might indicate its regulatory role in the early events of leaf senescence (Hinderhofer and Zentgraf 2001; Lim et al. 2003). Expression of *AtWRKY6* was also enhanced during leaf senescence and pathogen defense
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(Yoshida 2003). One of the targets of AtWRKY6 is the senescence-induced receptor-like kinase (SIRK) gene, which is expressed specifically in senescing leaves (Yoshida 2003). The expression of a senescence-associated receptor-like kinase (SARK) gene in bean (Phaseolus vulgaris) was delayed by light and cytokinin, but enhanced by darkness and ethylene, indicating the role of this gene in the regulation of leaf senescence (Hajouj et al. 2000; Lim et al. 2003). The SARK gene from Glycine max (GmSARK) has also been shown to be involved in regulation of leaf senescence (Li et al. 2006). SIRK and SARK genes have similar structures and belong to the leucine-rich repeat (LRR) class of RLK gene family (see Chapter 5, paragraph 5.2), which are key components in the perception of extracellular messages and subsequent phosphorylation cascades (Yoshida 2003). Similar to other known developmental programs in plants, senescence processes may also be regulated by signal transduction pathways (Hajouj et al. 2000). Post-transcriptional regulation of senescence has also been observed, such as protein modification by kinases and phosphatases in proteins responsible for nutrient remobilization processes. Several protein kinases exist in plants and are expressed during senescence and involved in executing the process (Hopkins et al. 2007).

3.4.1.3 Senescence-specific genes (SAG12 and SAG13)

As mentioned before, expression of SAGs are induced by different internal or external factors. Analysis of these genes has shown that there is some overlap between age-dependent senescence and senescence induced by other factors, although separate groups of senescence genes are induced under each induction factor (Lim et al. 2003; Weaver et al. 1998). These observations indicate the existence of a complex regulatory pathway in leaf senescence (Fig. 3-1). Some SAGs are involved in initiation, some in progression and some in both initiation and progression of the senescence process. For a better understanding of the roles of senescence-related genes, different investigators have classified these genes in different groups according to the time of their up- and down-regulations. Lohman et al. (1994) defined senescence genes in Arabidopsis in two main groups, SAGs and SDGs, and SAGs were further divided into two classes by Gan and Amasino (1997). Figure 3-2 illustrates the expression of these two classes of SAGs and also SDGs during leaf development (Gan and Amasino 1997). Lim et al. (2003) classified Arabidopsis senescence-related genes in six classes which also include regulatory genes that control expression of SAGs and SDGs. Similarly, Buchanan-Wollaston (2003) classified all senescence-related genes into four groups, including SAGs and SDGs, which showed change in their expression during different stages of leaf development. Although, a single gene can be in different classes, in all these classifications there are
some genes, which exclusively are expressed during senescence processes. One of these genes is SAG12 whose transcript is not detectable in non-senescing leaves (Lohman et al. 1994). Senescence-specificity of SAG12 has also approved in P_{SAG12}-GUS transgenic tobacco (Grbic 2002). The levels of SAG12 mRNA increase throughout the progression of senescence and reach maximal levels at the last stage of this process (Lohman et al. 1994). Therefore, SAG12 is reported as a senescence-specific gene. The SAG13 gene is also recognized as senescence-specific (Gan and Amasino 1997; Weaver et al. 1998).

\[ \text{Figure 3-2. Differential gene expression during leaf senescence. Expression of SDGs decreased in the onset of senescence while the expression of SAGs is increased. CAB: chlorophyll a/b binding protein gene, SSU: Rubisco small subunit gene (Gan and Amasino 1997).} \]

\textit{SAG12} and \textit{SAG13} are not only expressed in senescence-specific patterns, but also in an age-dependent manner (Gan and Amasino 1997). Transcripts of \textit{SAG12} are not detectable in young leaves of \textit{Arabidopsis} during the initial stages of detachment-, dark-, ethylene-, ABA- and dehydration-induced senescence, but slightly detected in old leaves in response to some of these induction factors (Noh and Amasino 1999; Weaver et al. 1998). In addition, transcripts of \textit{SAG12} are not detected in ozone-induced leaf senescence (Miller et al. 1999) but are present in ultraviolet-B (UV-B) treated plants (John et al. 2001). However, \textit{SAG13} responded strongly to ABA treatment in both young and old leaves (Weaver et al. 1998) as well as to ozone-induced leaf senescence (Miller et al. 1999) and to UV-B radiated plants (John et al. 2001). Therefore, \textit{SAG12} can be used as the best molecular marker for natural (age-dependent) senescence (Gan and Amasino 1997; Weaver et al. 1998). However, \textit{SAG12} and \textit{SAG13} genes are not leaf-specific and they are expressed in other tissues and organs such as stems, sepals, petals and carpels.
(Gan and Amasino 1997; Grbic 2002). Moreover, Grbic (2003) showed that the control mechanisms of SAG12 expression in leaf and stem tissues are different and the expression occurs in stems about two weeks later than in leaves. This might be because of the requirement of a functional stem for transporting nutrients from senescing leaves to developing seeds and flowers (Grbic 2003). The SAG12 gene encodes cathepsin L cysteine proteinase and is involved in protein turnover during senescence (Lohman et al. 1994; Weaver et al. 1998). Cathepsin cysteine proteases are active at acidic pH, and are assumed to be localized to lysosomes or vacuoles (Grbic 2003). The SAG13 gene encodes a short chain alcohol dehydrogenase (John et al. 2001).

It has been suggested that plants have the capability to measure the actual age of a leaf and also have another mechanism to evaluate the actual age of the whole plant (Hinderhofer and Zentgraf 2001). Low sugar content caused by changing the sink-source balance of the leaf might be a trigger for induction of the age-dependent SAGs (Noh and Amasino 1999; Yoshida 2003). Sugar content of the leaf is also influenced by other factors such as light conditions, N status and developmental stage of the leaf. Therefore, several internal and external factors integrate to control senescence (Nam 1997; Yoshida 2003). Treatment of Arabidopsis leaves with cytokinins (kinetin and BAP), auxin (IAA) and sugars (sucrose, glucose and fructose) repressed the accumulation of SAG12 transcript, which can consequently delay developmental senescence (Noh and Amasino 1999). None of these treatments repressed the expression of SAG13, indicating the existence of a distinct regulatory pathway for this gene (Noh and Amasino 1999). However, accumulation of sugars in P_{SAG12}-IPT transgenic tobacco plants during leaf senescence, due to the breakdown of accumulated starch or preferential export of N from the leaf, can block the retardation effect of cytokinin (Wingler et al. 1998). Transgenic tobacco plants with P_{SAG12}-GUS revealed activation of the SAG12 promoter in all cells of senescing leaves but only in specific domains of senescing flowers including corolla limb, corolla abscission zone, anthers and pistils (Grbic 2002). This indicated that the regulation of SAG12 promoter in flowers might not be conserved between tobacco and Arabidopsis (Grbic 2002). Further work on the SAG12 promoter revealed its conserved region, which is responsible for senescence-specific regulations (Noh and Amasino 1999). In general, these specific characteristics of the SAG12 gene make it unique to use in manipulation of senescence (particularly leaf senescence), which will be explained more in detail later on in this chapter (paragraph 3.4.3.2).
3.4.2 Biosynthesis of cytokinins

Naturally available cytokinins are adenine derivatives with an isoprenoid side chain (Kakimoto 2003). Precursors for the formation of these isoprene structures are either mevalonic acid or pyruvate plus 3-phosphoglycerate. These precursors are converted to the biological isoprene unit dimethylallyl diphosphate (DMAPP), which is used for cytokinin production (Taiz and Zeiger 2002). Depending to their side chain structure, cytokinins are classified as isoprenoid or aromatic cytokinins; the latter is very rare. Isoprenoid cytokinins are available in either an isopentenyladenine-type or zeatin-type cytokinin. Zeatin-type cytokinins is further divided to cis- and trans-zeatin forms. Trans-zeatin is more active in higher plants. Cytokinins are available in base, riboside or ribotide forms and only the base form is active and can be bound to cytokinin receptors. Cytokinin oxidases / dehydrogenases destroy cytokinins by cleaving the side chain (Kakimoto 2003).

3.4.2.1 Biosynthesis pathway in Agrobacterium

The first cytokinin biosynthesis gene was discovered in bacteria (Akiyoshi et al. 1984). The presence of some amounts of auxin and cytokinin in crown gall tissues indicated the existence of auxin and cytokinin biosynthesis pathways in these tissues. Crown gall tissues are produced on plants after infection by A. tumefaciens. Virulent strains of Agrobacterium contain a large plasmid known as Ti plasmid. The gene involved in cytokinin biosynthesis is located on a small portion of this plasmid called T-DNA, which is the part transferred and incorporated into the DNA of the host plant (see Chapter 4, paragraph 4.2.2). This gene, which is known as ipt (in contrast to plants, bacterial genes are written in lower case italics) encodes the isopentenyl transferase (IPT) enzyme. This enzyme transfers the isopentenyl group from DMAPP (isoprene unit) to adenosine moiety (AMP in bacteria and ADP and ATP in plants). This is the first and rate-limiting step in cytokinin biosynthesis pathway in both bacteria and higher plants. The isopentenylated side chain is then hydroxylated to form zeatin-type cytokinin (Akiyoshi et al. 1984; Kakimoto 2003; Taiz and Zeiger 2002). Figure 3-3 shows the biosynthesis of trans-zeatin in both plants and bacteria (Kakimoto 2003). Overexpression of the ipt gene in transgenic plants showed both the increased levels of cytokinin production and typical cytokinin responses in the host plants (Sun et al. 2003). The T-DNA also contains genes, which encode enzymes that convert tryptophan to IAA and genes for biosynthesis of nitrogen containing compounds known as opines (such as octopine and nopaline), which are responsible for tumor formation. All these genes have
plant eukaryotic promoters and therefore none of them is expressed in bacteria. They can only be expressed after integration into the plant DNA.

Figure 3-3. Cytokinin biosynthesis pathways in plants (indicated with bold arrows in the first step) and bacteria (with normal arrows). In Arabidopsis AtIPT1 has been reported that can also use AMP as adenosine moiety, but it uses ATP and ADP much more efficiently. Cytokinins in shade can bind directly to cytokinin receptors. An alternative pathway, where a hydroxylated side chain (HMBDP) can bind to the adenosine moiety, is shown in the bottom of the picture (Kakimoto 2003).

The ipt (or cyt) gene on T-DNA is also known as tmr because mutations in this gene result in “rooty” tumors. In the same way, genes involved in IAA biosynthesis are called tms (tms1 (or auxA or iaaM) and tms2 (or auxB or iaaH)) because mutants exhibit the
“shooty” tumors (Taiz and Zeiger 2002). Some strains of \textit{A. tumefaciens} contain another gene, which is called \textit{tzs} (\textit{trans}-zeatin-secretion) and is located on a Ti plasmid outside the T-DNA. This gene is responsible for a high level of cytokinin production in these strains (Kakimoto 2003).

3.4.2.2 Biosynthesis pathway in \textit{Arabidopsis}

Homologues of the \textit{Agrobacterium ipt} gene have recently been isolated from \textit{Arabidopsis thaliana}. Screening of the \textit{Arabidopsis} genome database for \textit{IPT} homologous showed nine genes coding for putative IPT enzyme, which are designated as \textit{AtIPT1-9} (Kakimoto 2003; Takei et al. 2001). With the exception of \textit{AtIPT2} and \textit{AtIPT9}, seven other \textit{Arabidopsis IPT} genes are able to catalyze the production of active cytokinin by encoding the cytokinin biosynthesis enzyme, which uses ATP and ADP preferentially as adenosine moiety and produce iP and \textit{trans}-zeatin (Kakimoto 2003; Sun et al. 2003). Recent studies have shown that \textit{AtIPT2} and \textit{AtIPT9} are responsible for the production of \textit{cis}-zeatin type cytokinin (Miyawaki et al. 2006).

The difference between plant and \textit{Agrobacterium} IPT enzymes is that IPT enzyme in \textit{Agrobacterium} uses adenosine monophosphate (AMP) as adenosine moiety whereas in plants, IPT enzyme preferentially uses ATP and ADP (Kakimoto 2003). The products of these reactions are iPMP (isopentenyladenosine - 5'- monophosphate), iPDP and iPTP and they finally are hydroxylated and converted to trans-zeatin (Kakimoto 2003; Taiz and Zeiger 2002). However, studies on \textit{AtIPT8} have shown that the IPT enzyme (\textit{AtIPT8}) encoded by this gene uses AMP as adenosine moiety for production of iPMP in plants (Sun et al. 2003). A similar reaction was observed for IPT enzyme encoded by \textit{AtIPT1} (Takei et al. 2001). Miyawaki et al. (2004) showed the tissue specificity and regulation of each \textit{Arabidopsis IPT} gene by using transformation of \textit{Arabidopsis} with GUS gene fused to the regulatory sequence of each \textit{AtIPT} gene. For example, \textit{AtIPT1::GUS} was expressed in xylem precursor cell files in root tips while \textit{AtIPT3::GUS} was expressed in phloem tissues (Miyawaki et al. 2004). The alternative pathway where a hydroxylated side chain (4-hydroxyl–3–methyl–2-(E)-butenyl diphosphate or HMBDP) is added to an adenosine moiety has been found in some strains of \textit{Agrobacterium} and this pathway might be also present in plants (Fig 3-3) (Kakimoto 2003). Sun et al. (2003) proposed that \textit{Arabidopsis}, and also other higher plants, might have several pathways for cytokinin biosynthesis.
3.4.3 Cytokinin overproduction in plants

Since the ipt gene from A. tumefaciens can catalyse the first and rate limiting step of cytokinin biosynthesis in higher plants, it can be used under control of suitable promoters in order to reduce leaf senescence. High levels of cytokinin can produce a range of physiological and morphological abnormalities in many plants such as reduced plant and leaf size, weakened apical dominance, less developed vascular and root systems, and can even induce cell death in some plant species (Carimi et al. 2003; Gan and Amasino 1996). Therefore, constitutive promoters like cauliflower mosaic virus (CaMV) 35 S are not suitable for this purpose (Gan and Amasino 1996). Several studies have been performed to express this gene in higher plants under the control of various promoters such as CaMV 35S, Cu²⁺–inducible, heat-shock inducible, light- inducible, wound-inducible, fruit-specific and development-specific promoters (Gan and Amasino 1996; Luo et al. 2005; Noh et al. 2004).

3.4.3.1 Cytokinin biosynthesis under control of heat shock promoter

Medford et al. (1989) fused a promoter region from a maize gene encoding a heat shock protein (HSP70) to the ipt gene from A. tumefaciens to make a P_HSP70-IPT construct for transformation of Arabidopsis and tobacco plants. Expression of heat shock (HS) genes is initiated in response to elevated temperatures and they encode proteins which protect biomolecules and organelles of plant cells from heat damage by enhancing the thermotolerance (Medford et al. 1989; Veerasamy et al. 2007). After heat shock treatment, the level of zeatin increased 52-fold, zeatin riboside (ZR) 23-fold and zeatin riboside 5’-monophosphate (ZMP) two fold in transgenic plants (Medford et al. 1989). However, under normal temperature, the levels of ZR and ZMP elevated 3 and 7 times, respectively, indicating the leakiness of the promoter. This increase in endogenous level of cytokinin under controlled temperature had some effects on transgenic plants: In tobacco, the height, xylem content and leaf size of the plant was reduced, axillary bud growth increased and an underdeveloped root system was observed, but no alteration in flowering time was reported. In Arabidopsis, time to flowering was not changed but a reduced root system was found. In both plants, the root tip region of the transgenic plants was wider than that of wild-type plants. Also the primary root elongated at slower rates compared to wild-type. Moreover, root hairs emerged closer to the root tip suggesting a reduction in elongation zone in the transgenic plants (Medford et al. 1989).

A similar experiment was carried out in tobacco using the ipt gene but under the control of the soybean heat shock promoter HS6871 (Smart et al. 1991). In the heat shock
area of the leaf, the level of zeatin was 17-fold higher, zeatin ribotid 7-fold higher and ZR 8-fold higher than in the rest of the leaf (Smart et al. 1991). Transformed plants grown at 20°C were shorter, had larger side shoots and remained green for a longer time than untransformed plants. However, after several heat shocks, the transformed plants exhibited an abundance of tiny shoots at the apex and a release of lateral buds. The different results compared to those reported by Medford et al. (1989) might be due to using different heat shock methods (Smart et al. 1991). However, in both cases the promoter showed leakiness.

3.4.3.2 Autoregulatory biosynthesis of cytokinin

Studies on differential gene expression during natural leaf senescence in *Arabidopsis*, lead to the discovery of the highly senescence-specific gene, SAG12 (Lohman et al. 1994). As explained earlier in this chapter, SAG12 gene is expressed in an age-dependent manner only during the onset of senescence (Gan and Amasino 1997; Lohman et al. 1994). Therefore, its promoter can be used to control the expression of the *ipt* gene to make an autoregulatory senescence-inhibition system. *Arabidopsis* SAG12 promoter was fused to the coding region of *ipt* gene from *A. tumefaciens* to form the $P_{SAG12}$-IPT construct (Fig. 3-4) and tobacco plants were transformed with this construct (Gan and Amasino 1995).

![pSG529(+)](pSG529_plus.png)

Figure 3-4. $P_{SAG12}$-IPT gene constructed by Gan and Amasino (1995) has been used for senescence retardation in many species including tobacco, broccoli and petunia.

Theoretically this promoter should activate the expression of *ipt* only at the onset of senescence, which leads to increase of cytokinin levels of the leaf and prevent senescence. The prevention of senescence would attenuate the promoter expression (due to its senescence-specificity) and consequently prevent cytokinin overproduction (Gan and Amasino 1995). This system has three important appearances: spatially, cytokinin production is targeted to leaf tissues; temporally, cytokinin production occurs only during senescence; quantitatively, cytokinin maintains at the minimum level necessary for senescence inhibition (Gan and Amasino 1996). It has also been reported later that
cytokinins can repress the expression of SAG12 (Noh and Amasino 1999; Weaver et al. 1998). However, this approach has not been successful for delaying fruit and flower senescence (Noh et al. 2004).

\[ \text{P}_{\text{SAG12}} \text{-IPT transgenic tobacco plants were identical to wild-type plants in growth and development except for a delay in leaf senescence. Their shoot and root systems were equally developed and both types had the same flowering time with the same numbers of nodes and same height. After 12 weeks both types had a similar number of flowers but at the later stages when senescence started in wild-type plant, the transgenic type had more than 300 flowers compared to about 180 flowers in wild-type. More experiments with detached leaves, reciprocal grafting, and photosynthesis measurements in \text{P}_{\text{SAG12}} \text{-GUS transgenic plants showed that in general there is no phenotypic developmental abnormality in transgenic plants, which proved the autoregulatory senescence-inhibition system (Gan and Amasino 1995; Gan and Amasino 1997). The only difference was in the progression of senescence in transgenic plants compared to wild-type. When wild-type plants aged, leaf senescence started from the bottom of the leaves and progressed to the top, while in leaves of transgenic plants no visible sign of senescence was observed at the identical age and development (Gan and Amasino 1995). Figure 3-5 shows the wild-type (left) and \text{P}_{\text{SAG12}} \text{-IPT transgenic (right) plants of tobacco at the same age (Gan and Amasino 1997). In general this system increased biomass and seed yield and enhanced postharvest longevity of leaves, and it may be possible to use this system in other crops as well (Gan and Amasino 1995). These transgenic plants were used later on to study the relationship between cytokinin, sugars and light during senescence, and this study showed that increased levels of sugars during senescence in transgenic plants can block the effects of cytokinin especially in low light. However, moderate light can reduce the effect of sugar repression (Wingler et al. 1998). Since the first use in tobacco, the strategy of \text{P}_{\text{SAG12}} \text{-IPT construct invented by Gan and Amasino (1995) was used for senescence retardation in many plant species (Chang et al. 2003; Chen et al. 2001; Clark et al. 2004; Jordi et al. 2000; McCabe et al. 2001; Schroeder et al. 2001). Jordi et al. (2000) transformed tobacco plants with this construct to study the effects of delayed leaf senescence and modified cytokinin biosynthesis on photosynthesis, protein levels of leaves and N partitioning of the plants. Plants were grown with growth limiting N supply. Results showed that amounts of zeatin and ZR in transgenic plants increased up to 15-fold in senescing leaves and two-folds in younger leaves compared to wild-type plants (Jordi et al. 2000). Older leaves of transgenic plants retained chlorophyll and turgor until flowering time. In contrast to the results obtained by Gan and Amasino (1995) where no} \]
change was observed in plant height, 5-10% reduction was observed in average plant height of transgenic plants. No further abnormalities were registered in transgenic plants (Jordi et al. 2000).

Figure 3-5. Retardation of leaf senescence by an autoregulatory senescence-inhibition system. Wild-type (left) and \( \text{P}_{\text{SAG12}}-\text{IPT} \) transgenic (right) plants of tobacco (\textit{Nicotiana tabacum} cv. Wisconsin 38) with same age were compared (Gan and Amasino 1997).

Contents of chlorophyll, soluble proteins and Rubisco in old leaves of transgenic plants were maintained at 100 %, 30 % and 15 % of respective levels in young leaves, while in wild-type plants the chlorophyll, soluble protein and Rubisco dropped dramatically. Compared to wild-type, accumulation of N in transgenic plants was higher in senescing leaves than in younger leaves leading to inverted canopy N profile in these plants. High levels of N in old leaves were due to high cytokinin levels which consequently increased sink activity in these leaves for nitrogen. This leads to a reduction in productivity and biomass of these transgenic plants under limited N because old leaves are less efficient in photosynthesis (Jordi et al. 2000).

Jasmine tobacco (\textit{Nicotiana alata}), an ornamental bedding plant, was transformed with \( \text{P}_{\text{SAG12}}-\text{IPT} \) construct for senescence retardation (Schroeder et al. 2001). Plants were grown under different fertilizer treatments for induction of leaf senescence. In general, transgenic plants exhibited 2- to 4- fold fewer senesced leaves and appeared more compact and dense compared to wild-type plants. Transgenic plants were 28 % shorter and had 26 % more shoot dry weight and had up to 174 % more branches, which indicated that the expression of SAG12 promoter is not tightly restricted to senescence time but is expressed sufficiently during plant development. Transgenic plants exhibited between 32 to 50 % reduction in number of flower per branch, which might be due to fertilizer stress. However, due to more flowers per branch in wild-type plants, the total number of flowers per plant was not significantly different between transgenic and wild-type plants. Transgenic plants showed 29 % increased in \textit{in situ} flower longevity.
general, these data provide new information about this construct and its potential for biological studies and agricultural applications (Schroeder et al. 2001).

Since leaf senescence is a problem in lettuce (*Lactuca sativa*), McCabe et al. (2001) transformed this plant with the PsAG12-IPT construct in order to solve the problem. In contrast to the PsAG12-IPT construct (Fig. 3-4) from Gan and Amasino (1995), a GUS gene as “P35S-GUS-intron-T35S” was added next to the right border of the T-DNA (McCabe et al. 2001). Similar to the methods adopted by Jordi et al. (2000), plants were grown under nitrogen limitation for induction of leaf senescence. The obtained transgenic lines responded differently. In general, outer leaves of transgenic lines (in both intact and harvested heads) retained their chlorophyll and remained greener compared to control plants (McCabe et al. 2001). However, similar to control they lost their turgor. This observation is in contrast with results of Jordi et al. (2000) in transgenic tobacco leaves, where leaves retained their turgidity. The contrasting results might be due to the genotype difference. Chlorophyll and protein content in lower leaves of transgenic lines were higher than in control plants. Other abnormalities were observed in some transgenic lines including smaller sized leaves, short seedlings, slight delay in bolting, severe delay in flower bud formation and panicle development, and thicker- and less green stems and panicle branches (McCabe et al. 2001). However, no differences were observed between transgenic and control plants in head radius, height or fresh weight. Transgenic flowering plants showed a large increase in IPA riboside, ZR and DHZ riboside in their upper parts compared to control plants. Growth rate, total nitrate and total nitrogen dropped in the entire transgenic and control plant, when they were grown in absence of nitrogen. However, lower leaves of transgenic plants remained greener than control plants in response to this limiting condition. In general, advantages of PsAG12-IPT transgenic lettuce were a significant delay in senescence, an increase in postharvest performance of mature heads, a significant reduction in susceptibility to infection by *Botrytis cinerea*, and lower nitrate and nitrogen content under nitrogen deficiency conditions. The disadvantage of PsAG12-IPT transgenic lettuce was the delay in later developmental stage including bolting and flowering compared to wild-type (McCabe et al. 2001).

Transgenic plants of broccoli (*Brassica oleracea* var. *italica*) with PsAG12-IPT and also with PsAG13-1-IPT constructs for senescence retardation were obtained (Chen et al. 2001). Chlorophyll retention on detached leaves and florets was used as an indicator of retardation of yellowing. As result, 31 % of transgenic plants showed senescence retardation in detached leaves, 16 % in detached florets and 7.2 % in both detached leaves and florets. Transformation with PsAG12-IPT had a distinct impact on senescence
retardation of leaves, while the $P_{\text{SAG13-1}}$-IPT gene had a clear effect on delaying senescence of florets. Apart from senescence retardation, few abnormalities such as changes in time of bolting and number of floret branches were observed in some transformants. Also, most of the florets with delayed senescence were not uniform in color as they had green and yellow (chimeric) sections. In general, selection of suitable transgenic lines with delayed senescence in broccoli seemed feasible (Chen et al. 2001).

Petunia ($Petunia \times hybrida$ cv. V26) plants were also transformed with $P_{\text{SAG12}}$-IPT construct mostly for retardation of flower senescence (Chang et al. 2003). Transgenic lines showed delayed leaf senescence and increased flower longevity. The first visual symptom of flower senescence is corolla wilting which accelerated by pollination. Corolla wilting in transgenic flowers was observed 6-10 days later than in wild-type flowers. Expression of $ipt$ gene was up-regulated after pollination and reached up to 60-fold 48 hours after pollination. This leads to an accumulation of cytokinin (mostly zeatin and ZR) in transgenic corollas (Chang et al. 2003). Delay in corolla senescence of detached petunia flowers using cytokinin application has been reported previously (Taverner et al. 1999). In addition to the above-mentioned changes, transgenic flowers exhibited both a delay in ethylene production and retardation in ethylene sensitivity compared to wild-type flowers. Expression studies of the gene encoding ACC oxidase (ethylene biosynthesis gene) confirmed that induction of this gene by pollination was delayed in transgenic flowers. In addition, while treatment with ethylene did not change the cytokinin content of wild-type corollas, it resulted in 50-fold increase in cytokinin levels in transgenic ones. Transgenic corollas also exhibited a lower increase in ABA levels after pollination compared to wild-type corollas (Chang et al. 2003). Similar to previous reports (Schroeder et al. 2001), expression of the SAG12 promoter was not tightly regulated since $ipt$ transcript was detected in non-senescing corollas. In addition, some abnormalities such as decreased adventitious rooting and increased branching were observed. In general, these transgenic petunias provided information about interaction among cytokinins, ethylene and ABA hormones (Chang et al. 2003).

In another experiment with these transgenic petunias, leaf senescence was induced by drought stress to study the horticultural performance of transgenic plants in relation to some important economical traits (Clark et al. 2004). Since a wide range of plant phenotypes was observed in the transgenic plant lines, two lines were selected, which exhibited a similar morphology as wild-type plants. Drought stress up-regulated the expression of $ipt$ in transgenic but not in wild-type leaves. Lower leaves showed a 53- and 65-fold increase in $ipt$ mRNA in the two different lines (Clark et al. 2004). However,
detection of *ipt* transcripts in non-senescing leaves showed that the SAG12 promoter is not senescence-specific in petunia (Clark et al. 2004). Chlorophyll content of lower leaves of transgenic lines was also retained seven days after stress. No significant difference was observed in time of flower induction between transgenic plants and WT, which is in agreement with results of Gan and Amasino (1995) in tobacco plants. One of the transgenic lines had higher numbers of total branches per plant and it also showed a decrease in adventitious rooting compared to wild-type, whereas the other line was more similar to wild-type regarding these traits. In general, this study showed that retardation of leaf senescence using this construct is possible in petunia, but some abnormalities might also be generated (Clark et al. 2004).

The use of this construct for transformation of other species such as cauliflower, tomato and *Arabidopsis* also has been briefly reported but insufficient information is available about the results. To my knowledge, no study on using this construct for transformation of woody plants has been reported. In the present PhD project, miniature potted rose (*Rosa hybrida* cv. Linda) was transformed with the P<sub>SAG12</sub>-IPT construct made by Gan and Amasino (1995) for reducing leaf senescence (Manuscript). Transgenic shoots exhibited a decrease in adventitious root induction compared to control plants. They also remained green for longer period compared with control shoots, under *in vitro* condition without sub-culturing into fresh media. Shoots of one of these lines were finally rooted and transferred to the greenhouse for further phenotypical analysis. However, due to the time limit of this PhD study, analysis of these plants has not been finished at time of submission of the thesis. Latest results on these plants are reported in the manuscript (page 95) and general discussion.
4. Genetic transformation of *Rosa hybrida*

Production of new cultivars of ornamental plants is driven by grower and customer demands for new traits such as resistance to environmental stress, pests and diseases, longer vase life, leaf and flower longevity, plant morphology and new flower form, color and scent (Tanaka et al. 2005). Conventional breeding methods for introducing new genes into plants are both time consuming and constrained by some restrictions such as gene pool limitations. Additionally, many flowering plants are propagated vegetatively, which further limits the available gene pool (Robinson and Firoozabady 1993). For example in rose: high degree of inter-cultivar sterility due to different ploidy levels, its perennial and woody nature, high degree of heterozygosity and lack of inbred lines are problems for conventional breeding (Firoozabady et al. 1994). The problem with a limited gene pool can be overcome by using genes from other plant species or even other organisms (Slater et al. 2003; Tanaka et al. 2005). However, gene transfer techniques are a prerequisite for the application of the recombinant DNA or new genes from other species to the new cultivar (Robinson and Firoozabady 1993). New biotechnological methods, like biolistic or *Agrobacterium*-mediated transformation, can provide a way to introduce a desired gene into established cultivars (Hansen and Wright 1999).

The genetic transformation of plants depends on the ability of successful transformation at the cellular level and successful plant regeneration from the transformed cells (Hansen and Wright 1999; Li et al. 2002; Robinson and Firoozabady 1993). Successful transformation, however, depends on several factors such as plant species, cultivar, explant type, expression of a selection gene and transformation method. In general, some species are easy to transform while others such as cereals, legumes and many woody plants are recalcitrant for transformation (De la Riva et al. 1998). Among different transformation methods, the most successful method has been cocultivation of regenerable explants with *Agrobacterium* strains (Robinson and Firoozabady 1993). However, in the present study both biolistic and *Agrobacterium*-mediated transformation were used for introducing the \( P_{SAG12}\text{-IPT} \) gene (see Chapter 3) into somatic embryos of miniature potted rose, *R. hybrida* ‘Linda’ (Manuscript) and therefore, I will focus more on these two methods here.

4.1 Direct gene transfer methods

Since *Agrobacterium* naturally infects only dicotyledonous plants, direct gene transfer methods were established and mainly used for transformation of monocots (De la Riva et
al. 1998). However, today *Agrobacterium* strains can transform a broad range of organisms including monocotyledonous plants (De la Riva et al. 1998; Gelvin 2003; Slater et al. 2003). Direct plant transformation systems are known to be disturbing to the cells, expensive and poorly reproducible due to variable transgene copy-number per genome (De la Riva et al. 1998). These methods includes polyethyleneglycol-mediated gene transfer, laser-mediated uptake of DNA, microinjection, ultrasound and *in planta* exogenous application, protoplast and intact cell electroporation and particle bombardment or gene gun technology (De la Riva et al. 1998; Slater et al. 2003). The later one, particle bombardment, which is also called biolistic, is the major direct gene transfer method, which is used in many laboratories (Slater et al. 2003). Since this method was used in the present study, I will explain it here briefly.

### 4.1.1 Biolistic transformation technique

This method is the most important and most effective method among other direct gene transfer methods (Slater et al. 2003). In summary, gold or tungsten particles (microcarrier) are coated with plasmid DNA, which contains the gene of interest or marker gene and is then used for bombardment of the target plant material. Using this method, the integration of the transgene in the genome occurs infrequently. However, the transformation is independent of species, cultivar and explant type (Marchant et al. 1998b). Optimisation of the system is very crucial for efficient transformation and focuses on three aspects of the process: particle type and preparation, particle acceleration and choice of target material. Particle number is important since too little DNA may lead to low transformation frequency and too much DNA may lead to high copy number and rearrangement of the transgene (Slater et al. 2003). For example, reducing the amount of particles led to a significant increase in transient expression levels of inserted genes in *Arabidopsis* and tobacco (Helenius et al. 2000). Plant materials used for biolistic transformation are either non-embryogenic explants which are bombarded and then induced to become embryogenic or are embryogenic cultures (Slater et al. 2003). The pressure for bombardment can be provided by gun power, by gases such as helium or CO$_2$, or by an electric discharge (Hansen and Wright 1999). In old systems a vacuum chamber is used while in new methods a gene gun is used without vacuum and therefore the system can also be used in the field. The vacuum chamber method provides a more controlled bombardment environment, while the Helios$^{\text{TM}}$ Gene Gun makes it possible to select a wide range of target material including plants in the field (Helenius et al. 2000).
4.1.2 Biolistic transformation of *Rosa hybrida*

Transformation of rose by the biolistic method has been reported previously (Marchant et al. 1998a; 1998b). Embryogenic callus of Floribunda rose, *R. hybrida* ‘Glad tidings’ were used for transformation with GUS and *nptII* genes (see paragraph 4.3.1) by using the vacuum chamber system in order to develop a reproducible biolistic transformation method (Marchant et al. 1998b). Transformation was successful and Southern analysis confirmed the presence of 1-5 copies of the *nptII* gene into the rose genome (Marchant et al. 1998b). Similar transformation was carried out in order to confer resistance to blackspot disease into the above-mentioned cultivar (Marchant et al. 1998a). The rice basic (Class I) chitinase gene under the CaMV 35S promoter and also *npt II* gene as selectable marker were used for this study. Transformation was successful and 2-6 copies of the transgene were integrated into the genome of different transgenic lines. Bioassay analysis showed that expression of the chitinase gene reduced the development of blackspot by 13-43% (Marchant et al. 1998a).

In the present study, embryogenic callus of miniature-potted rose, *R. hybrida* ‘Linda’ was used as explant for transformation using biolistic method. Three different helium pressures including 100, 150 and 200 psi (100 psi = 6.9 bar) were used as treatments in the present study. Unfortunately no transgenic callus was obtained. Since two running preliminary experiments were also ended without results, this transformation method was not used any further.

4.2 *Agrobacterium* - mediated transformation

Since in this method a micro-organism is used for gene transfer into plant cells, it is called indirect gene transfer system (Dohm 2003). Viral vectors have also been used for indirect gene transfer into plant cells. However, only *Agrobacterium*-mediated transformation is presented here.

4.2.1 *Agrobacterium* genus

*Agrobacterium* is a soil-borne, rod shaped and gram-negative pathogenic bacterium, which is found in the rhizosphere and can transfer DNA into a broad group of organisms including monocots and dicots, gymnosperms, yeasts, ascomycetes, basidiomycetes and even human cells (Gelvin 2003; Slater et al. 2003). The *Agrobacterium* genus has been divided into several species according to host range and disease symptoms. According to this classification, *A. radiobacter* is avirulent, *A. tumefaciens* causes crown gall disease,
A. rhizogenes causes hairy root disease, A. rubi causes cane gall disease and A. vitis causes galls on grape and a few other plant species (Gelvin 2003). However, A. tumefaciens can be converted into A. rhizogenes by replacing the tumour-inducing (Ti) plasmid with rhizogenic (Ri) plasmid. Therefore, this classification does not seem to be correct (Gelvin 2003).

One of the advantages of transformation using Agrobacterium strains rather than direct transformation methods is that it comprises less rearrangement of the transgene and results in a lower transgene copy number, which leads to fewer problems with transgene co-suppression (gene silencing) and instability (De la Riva et al. 1998; Slater et al. 2003). Moreover, due to the fact that this method is a single-cell transformation system, mosaic plants will not be produced (De la Riva et al. 1998).

4.2.2 A. tumefaciens and Ti plasmid

Plant transformation using Agrobacterium strains has become the most used method for introducing a new gene into plant cells (De la Riva et al. 1998; Slater et al. 2003). This method is based on the special ability of this bacterium to transfer a part of its tumor-inducing (Ti) plasmid, which is called T-DNA, into the genome of the host cell (De la Riva et al. 1998). The Ti plasmid is very big, has a size between 200 and 800 kbp (Gelvin 2003), and contains one or more T-DNA regions (Slater et al. 2003). The T-DNA contains three different gene loci responsible for biosynthesis of cytokinin and auxin (see Chapter 3, paragraph 3.4.2.1) and nitrogen containing compounds known as opines (amino acid derivatives) and agropines (sugar derivatives), which are used by A. tumefaciens as carbon and energy source (Slater et al. 2003). Genes responsible for production of auxin (tms1 and tms2) and cytokinin (ipt or tmr) are often referred to as oncogenes, because they are the first determinants of tumour phenotype (Slater et al. 2003).

In A. rhizogenes the big plasmid containing T-DNA, is called Ri plasmid because it induces hairy roots after integration and expression in the genome of a host plant (De la Riva et al. 1998). The T-DNA is also referred to as T-region on both Ti and Ri plasmids and it is between 10-30 kbp on native plasmids (Gelvin 2003). The genes, which are responsible for the T-DNA transfer from the bacterium into the plant cells are called vir genes and are located on the Ti plasmid vir (virulence) region outside the T-DNA. However, they have cooperation with the genes from bacterial chromosome for this action (De la Riva et al. 1998; Gelvin 2003). The vir region consists of 8 operons designated virA-H and the number of the genes per operon is different (De la Riva et al.
1998). It has been shown that any foreign DNA located between the T-DNA borders can be transferred into the plant cells (De la Riva et al. 1998). Therefore, this ability is used to make vector and bacterial strain systems for plant transformation (De la Riva et al. 1998; Hellens et al. 2000).

With the basis that the vir genes are able to transfer T-DNA from other plasmids into the host genome, the binary vector system was invented (Hellens et al. 2000). In this system, a Ti plasmid known as helper Ti plasmid (Fig. 4-1.A), containing vir genes but without T-DNA region is placed into an Agrobacterium strain together with a main vector (Fig. 4-1.B) containing a gene of interest between the right and left borders, but without vir genes. Figure 4-1 illustrates the plasmid containing vir genes and the plasmid containing the PsAG12-IPT construct, which has been used in the present study. Antibiotic resistance encoding genes are usually used in the Ti plasmid on the T-DNA or outside the T-DNA as effective selectable markers to facilitate the selection of transformed explants or bacteria, respectively (Slater et al. 2003).

![A] Ti plasmid with vir region but without T-DNA. This plasmid is used in binary vector system for transferring T-DNA from the main vector into the host genome. 

![B] main vector without vir genes and containing PsAG12-IPT gene together with kanamycin resistant gene for plant selection between right and left borders of T-DNA and gentamycin resistant gene for selection of transformed Agrobacterium. Names of restriction enzymes have also mentioned to be used for further analysis of the transgenic plants.

4.2.3 Mechanism of T-DNA translocation

The mechanism of T-DNA transfer and integration into the host genome has been reviewed elsewhere (De la Riva et al. 1998; Gelvin 2003; Slater et al. 2003). In brief, signal molecules like sugars (such as glucose, galactose and xylose) and phenolic compounds (such as acetosyringone), which are released from the wounded plant, are
detected by VirA transmembrane protein (De la Riva et al. 1998; Slater et al. 2003). Artificial wounding or adding acetosyringone is usually done during the transformation procedure. However, these treatments are not compulsory when embryogenic callus or somatic embryos are used as explants (Dohm 2003). Autophosphorylated VirA has the capacity to transphosphorylate the cytoplasmic DNA binding protein VirG and phosphorylated VirG will function as transcriptional factor regulating the expression of other *vir* genes. The activation of *vir* genes produces the generation of single-strand (ss) copies of bottom T-DNA strand. Any DNA placed between borders of T-DNA will be transferred into the plant genome as single-strand. Different studies on T-DNA borders have shown that the strand synthesis starts from right border. Right borders of T-DNA initially appeared to be more important than left borders. However, synthesis can also be initiated from the left border but with very low efficiency. This is the result of presence of an enhancer or overdrive sequence next to the right border, which is recognized by VirC1 protein (De la Riva et al. 1998; Gelvin 2003; Slater et al. 2003).

The T-DNA strand is transferred into the plant cell as ssT-DNA-VirD2 complex. This complex is coated with VirE2 protein, which facilitates the translocation of the complex into nucleus. Different Vir proteins are participated in this translocation (review in details by De la Riva et al. 1998 and Gelvin 2003). After entering the cell, the ss-T-DNA-VirD2
complex targeted to the nucleus for integration of T-DNA into plant genome (De la Riva et al. 1998; Gelvin 2003; Slater et al. 2003). The process of integration of the T-DNA into host plant genome is called ‘illegitimate recombination’ (Slater et al. 2003). The mechanism of integration has not been characterised completely (De la Riva et al. 1998; Gelvin 2003). The mechanism of transferring the T-DNA from the Ri plasmid of A. rhizogenes into the host genome is similar to that by A. tumefaciens. Fig. 4-2 illustrates the role of some vir proteins in T-DNA translocation (http://boneslab.chembio.ntnu.no/Bi211/Bi211Kap8-03.html).

4.2.4 Factors affecting transformation with A. tumefaciens

As the Agrobacterium-mediated gene transfer is a complex process, the optimization of this process is very important for a successful transformation at high efficiency. As a general procedure, first Agrobacterium colonies must be cultured for overnight in a growth media at certain temperature (27-28 °C). Two known media for bacterial culture are Luria-Bertani (LB) and YEP media (Sambrook and Russell 2001). Then, Agrobacterium culture will be diluted in fresh LB or YEP media or another media such as MS (Murashige and Skoog 1962) or Minimal-A (Min-A) (Ausubel et al. 2002) media to reach a certain concentration. Further preparation of the bacterial suspension (dilution in different media) has affected the transformation efficiency (Dohm 2003). Different media have been used in rose transformation for dilution of the Agrobacterium cultures including YEP (Dohm et al. 2001), MS (Derks et al. 1995; Li et al. 2002) and Min-A (Dohm et al. 2001; Firoozabady et al. 1994). In the present study, YEP, LB, Min-A and ½ MS media were used (Manuscript). Explants are then inoculated with Agrobacterium culture, and subsequently cocultivated for different time periods. After this period, explants will be washed with antibiotics and then be cultured on selection media. Transformation protocols are different from one species to the other and within species, from one cultivar to the other (De la Riva et al. 1998). There are several factors, which have to be considered for optimization of transformation.

4.2.4.1 Agrobacterium strains

Several strains of Agrobacterium are available for plant transformation (Hellens et al. 2000; Robinson and Firoozabady 1993; Slater et al. 2003). For recalcitrant plant species, the choice of strain is a major factor contributing to the success or failure of the transformation, and instead of standard strains (like LBA4404) they may require so-called supervirulence strains such as EHA101 or EHA105 (Slater et al. 2003). In a ternary transformation system, Agrobacterium strains are supplemented with a plasmid
containing a constitutive \textit{virG} mutant gene (\textit{virGN54D}), which increases the efficiency of transformation and induces \textit{vir} genes in absence of acetosyringone (Van der Fits et al. 2000). Different strains including LBA4404, AGL0, EHA101, EHA105, C58C1, GV3101 and GV2260 have been used in transformation of roses (paragraph 4.2.4.4). In the present study, GV3850, AGL1 and LBA4404 strains were used (Manuscript).

4.2.4.2 Explant type

The type of explant is an important fact and it must be suitable for regeneration allowing the recovery of whole transgenic plants (De la Riva et al. 1998; Slater et al. 2003). Wide variety of explants have been used for transformation of ornamental crops including leaves, shoot tips, stems, petioles, pollen, filaments, petals, peduncles, corms, bulb scales, somatic embryos and friable embryogenic callus (Robinson and Firoozabady 1993). Similarly, different types of explants have been used for regeneration of whole plant in different rose cultivars (see Chapter 2). Since efficiency of many of these regeneration methods is very low and most of them are cultivar dependent, they are not suitable to use for transformation of rose cultivars (Robinson and Firoozabady 1993). Proliferating somatic embryos are suitable targets for transformation because the origin of proliferating embryogenic tissues is at or near the surface of the older embryos and is readily accessible to DNA delivery (Hansen and Wright 1999). Several studies have shown that embryogenic tissues and somatic embryos are among best materials for transformation of rose cultivars (Condliffe et al. 2003; Firoozabady et al. 1994; Rout et al. 1991; Van der Salm et al. 1996). In summary, different explants including stem slices (Van der Salm et al. 1997), embryogenic callus (Condliffe et al. 2003; Derks et al. 1995; Firoozabady et al. 1994; Kim et al. 2004; Li et al. 2002) and somatic embryos (Dohm et al. 2001) have been used for successful transformation of different rose cultivars. Embryogenic callus containing somatic embryos has been used as explant for transformation in the present study (Manuscript).

4.2.4.3 Inoculation time

Inoculation time varies from a few minutes to several hours and it is dependent on plant species, type of explant, \textit{Agrobacterium} strain and its concentration in the media which is measured as optical density at 600 nm (OD\textsubscript{600}). The concentration at OD\textsubscript{600} = 0.2-1.0 is normally used for inoculation. Embryogenic callus of different rose cultivars have been inoculated with \textit{Agrobacterium} suspension for different periods including 15 minutes (Derks et al. 1995), 30 minutes (Kim et al. 2004; Li et al. 2002), one hour (Dohm et al. 2001) and even two hours (Condliffe et al. 2003). In the present study, embryogenic
cultures of rose inoculated for about one hour with suspension of all *Agrobacterium* strains (GV3850, AGL1 and LBA4404) at OD$_{600} = 0.8-1.0$ (Manuscript).

4.2.4.4 Cocultivation period

Cocultivation period is about 2-6 days and dependent on the strain of the *Agrobacterium* (Dohm 2003), plant species, explant type and size. Cocultivation with more virulent *Agrobacterium* strains like EHA101 and EHA105 and strains supplemented with extra copies of *virG* or *virE1* genes needs shorter periods than with other strains. Cocultivation is usually carried out in the dark. Firoozabady et al. (1994) cocultivated embryogenic tissues of rose with LBA4404 and EHA101 strains for three days. The same period of time were used for cocultivation with C58C1 and AGL0 strains of *A. tumefaciens* (Derks et al. 1995). However, Condliffe et al. (2003) used two days for cocultivation with AGL0 strain. Van der Salm et al. (1997) and Li et al. (2002) also used two days for cocultivation of explants with GV3101 strain. While somatic embryos of rose cocultivated with EHA105 strain for two days, same explants cocultivated with GV2260 strain for six days (Dohm et al. 2001). In the present study, embryogenic tissues were cocultivated with AGL1, GV3850 and LBA4404 strains for two days (Manuscript).

4.2.4.5 Temperature

The activation of the vir system depends on the temperature and it will not express at temperatures higher than 32°C due to conformational changes in the folding of VirA, which inactivate its properties (De la Riva et al. 1998). The induction of *vir* genes is maximal at approximately 25 to 27°C (Gelvin 2003). However some strains are more stable at lower temperatures and it might be better to cocultivate the explant with those strains at lower temperatures (Gelvin 2003).

4.2.4.6 Antibiotics

Different antibiotics are used for either selection of transgenic explants or for washing the *Agrobacterium* after cocultivation period. However, high concentration of these antibiotics can decrease the regeneration efficiency (Li et al. 2002). Different concentrations of antibiotics, which have been used in transformation of rose cultivars, are summarized in Table 4-1. Cefotaxime, carbenicillin and vancomycin have been used most frequently. However, Kim et al. (2004) used 250 mg l$^{-1}$ Clavamox (amoxicillin trihydrate) for both washing the explants and in the media after cocultivation. In an investigation with different concentrations of kanamycin, carbenicillin, cefotaxime and
Literature review

Genetic transformation of Rosa hybrida

Combinations of carbenicillin and cefotaxime on shoot regeneration of R. hybrida ‘Carefree Beauty’, Li et al. 2002 showed that carbenicillin in any concentrations (250 and 500 mg l\(^{-1}\)) inhibited shoot regeneration from different types of explants. Different concentrations of cefotaxime (250 and 500 mg l\(^{-1}\)) showed negative effect on callus induction from leaf tissues, but after callus induction, only 500 mg l\(^{-1}\) concentration inhibited callus differentiation. However, successful regeneration of R. hybrida ‘Royalty’ on media containing 500 mg l\(^{-1}\) carbenicillin (Firoozabady et al. 1994) suggests that this effect of carbenicillin is genotype-dependent (Li et al. 2002).

Table 4-1. Names and concentrations of some antibiotics which have been used for killing the Agrobacterium after cocultivation and for preventing Agrobacterium overgrowth in some rose transformation studies.

<table>
<thead>
<tr>
<th>Antibiotics (mg l(^{-1}))</th>
<th>References</th>
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<tr>
<td>Cefotaxime</td>
<td>Carbenicillin</td>
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<tr>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>200</td>
<td>-</td>
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<td>500</td>
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<td>250, 500**</td>
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<tr>
<td>200</td>
<td>-</td>
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<tr>
<td>500</td>
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* Timentin (mixture of ticarcillin disodium and potassium clavulanate in 15:1 ratio) was used about three weeks after transformation for inhibiting the bacterial growth. It was not used for washing the Agrobacterium from explants.
** 250 mg l\(^{-1}\) was used for washing leaf explants while 500 mg l\(^{-1}\) was used for washing both undifferentiated callus and embryogenic callus.

4.3 Analyses of putative transformed plants

After transformation, transgenic tissues must be recognised and the presence of the transgene has to be confirmed using different methods. In this process, the first stage is selecting putative transgenic tissues using selectable marker genes on the media containing selection agent. However, marker genes do not exist in some transformation processes such as using native A. rhizogenes strains or using marker-free strategies. In such situations as well as in cases where marker genes are present, the existence of the transgene has to be confirmed with other methods. Some of these methods will be described here.
4.3.1 Marker genes

The first way for selecting the putative transgenic explants is using marker genes. Marker genes in general include two groups of genes: selectable marker genes and reporter genes (Chawla 2002). Selectable marker genes are delivered together with the gene of interest to identify and encourage the growth of recipient cells (Hansen and Wright 1999). They are usually located near to the left border of T-DNA (Hellens et al. 2000) and confer resistance to selection agents such as antibiotics or herbicides. Several selectable markers are available and among them neomycin phosphotransferase II (\textit{npt II}) is the most widely used marker. NPT II inactivates some antibiotics such as kanamycin, neomycin, geneticin and paromycin. Other selectable markers are hygromycin phosphotransferase (\textit{hpt}), gentamycin acetyltransferase, streptomycin phosphotransferase and herbicide resistant genes (Chawla 2002). NPT II protein can also be detected by using Enzyme-linked immunosorbent assay (ELISA).

However, antibiotics have negative effects on proliferation and differentiation of plants and also have negative effects to the environment. Therefore, different strategies for producing marker-free transgenic plants have been developed which use co-transformation strategies, site-specific recombination systems, transposable elements or intrachromosomal recombination (Dohm 2003). Different concentrations of kanamycin including 5 mg l\(^{-1}\) (Van der Salm et al. 1997), 60 mg l\(^{-1}\) (Dohm et al. 2001; Kim et al. 2004), 100 mg l\(^{-1}\) (Condliffe et al. 2003; Li et al. 2002), 250 mg l\(^{-1}\) (Marchant et al. 1998a) and 300 mg l\(^{-1}\) (Derks et al. 1995; Firoozabady et al. 1994) has been used in rose transformation. In the present study, different kanamycin concentrations were used in a preliminary experiment in order to obtain the optimum concentration and finally the concentration of 100 mg l\(^{-1}\) was used for the main experiment (Fig. 4-3, Manuscript). However, 60 mg l\(^{-1}\) kanamycin was used in biolistic transformation of the present study.

Figure 4-3. Selection of putative transgenic shoots of \textit{Rosa hybrida} ‘Linda’ transformed with P\textsubscript{SAG12}-IPT on kanamycin containing media. Control plants (left) and transgenic plants (right) one month after growth on 100 mg l\(^{-1}\) kanamycin containing MS media.
A second group of marker genes are reporter genes which include \textit{uidA} gene encoding β-glucuronidase (GUS) from \textit{E. coli}, \textit{luc} gene encoding luciferase (LUC) from firefly, a gene encoding green fluorescent protein (GFP) from jellyfish and some other genes such as opine synthase (\textit{ocs}), nopaline synthase (\textit{nos}) and anthocyanin regulatory genes (Chawla 2002). Among them \textit{uidA} (GUS) is the most frequently used reporter gene in plant transformation. In rose, transformation with GUS has been used in most of the experiments. However, in some rose studies other reporter genes including LUC (Firoozabady et al. 1994) and GFP (Kim et al. 2004) have been used. In the preliminary experiments of the present study, a P\textsubscript{SAG12}-GUS construct was used for transformation, but because of lack of embryogenic callus, further experiments were carried out only with P\textsubscript{SAG12}-IPT construct.

4.3.2 Using PCR

The easiest method for molecular detection of the transgene is PCR analysis (Dohm 2003). PCR can be used not only for detecting the gene of interest but also for the selectable genes (like \textit{nptII}) and reporter genes (\textit{GUS}, \textit{LUC} or \textit{GFP}). Thereby it is possible to confirm the existence of the transgene, and at same time prove whether the whole construct has been integrated into the host genome or only parts of it.

4.3.3 Southern blot analysis

This method can be used for detecting both transgenic plants and the number of integrated gene copies in the genome (Dohm 2003). As a high copy number of the gene may cause gene silencing, awareness of the copy number is an important characteristic in combination with further expression analysis of transgenic lines. Southern blot can be done with either radioactive or non-radioactive methods, and the latter one was used in the present study (Manuscript).

4.4 \textit{Agrobacterium} - mediated transformation of rose (\textit{Rosa hybrida})

Successful transformation of rose carried out for the first time in a cut rose, \textit{Rosa hybrida} cv. Royalty (Firoozabady et al. 1994). Friable embryogenic tissues of this cultivar were cocultivated with either \textit{A. tumefaciens} or \textit{A. rhizogenes} strains containing \textit{nptII}, \textit{GUS} or \textit{LUC} genes. The transformation efficiency was high and kanamycin resistant callus was obtained at the same frequency from experiments with both \textit{A. tumefaciens} and \textit{A. rhizogenes}. Transgenic plants were obtained and they flowered normally (Firoozabady et al. 1994).
Embryogenic callus of cut rose cultivars ‘Melody’, ‘Tineke’ and ‘Sonia’, were used for transformation via *A. tumefaciens* (Derks et al. 1995). Since high concentrations of microorganisms, especially bacteria in the vase water, block the vascular bundles of cut flower stems, resulting in wilting and bent-neck, genes encoding for antibacterial activity together with *nptII* and *GUS* genes were used in this study. Transgenic callus was obtained from all cultivars. However, only transgenic somatic embryos of ‘Sonia’ were obtained from transgenic embryogenic callus. No information on further development of the somatic embryos has been reported (Derks et al. 1995).

Transgenic plants of *R. hybrida* cv. Moneyway (rootstock) were produced using *A. tumefaciens* strain which contain a *ROLA, B* and *C* gene from *A. rhizogenes* (Van der Salm et al. 1997). *ROL* genes are involved in the observed changes of root formation and root morphology in the hairy root disease resulting from infection by *A. rhizogenes*. Three binary vectors were used containing *GUS, ROLB* and *ROLA, B, C* genes all together with *nptII* gene. In the first step, transgenic adventitious roots were produced on stem slices and in the second step these roots were used for induction of somatic embryogenesis and regeneration of transgenic plants. As result, first step was efficient, but the second step was less efficient which suggested the negative effect of kanamycin on formation of embryogenic callus. Transformation was successful and cuttings of transgenic plants showed 3-fold increase in adventitious root formation (Van der Salm et al. 1997). One of these transgenic lines was used for a grafting experiment with the cut rose cultivar ‘Madelon’ as scion (Van der Salm et al. 1998). Resulting plants showed the stimulation of both root development in rootstock ‘Moneyway’ and axillary shoot production in ‘Madelon’, which was untransformed. Since the number of axillary shoots at the base of the rose plant is correlated with flower production, these plants are useful for more efficient flower production (Van der Salm et al. 1998).

Two garden rose cultivars, *R. hybrida* ‘Heckenzauber’ and ‘Pariser Charme’ were transformed using *A. tumefaciens* strains, containing *nptII* and *nptIII* genes for kanamycin resistance and combinations of genes encoding different antifungal proteins. These genes were used to obtain partial resistance to fungal diseases including blackspot, powdery mildew, downy mildew and rust simultaneously. Somatic embryos were used as explants and transgenic plants of both cultivars were obtained. An average of 60% reduction in susceptibility against blackspot was obtained in some transgenic lines. However, some abnormalities in number of leaflets per leaf and number of petals in the flower as well as in male fertility were observed in several transgenic plants (Dohm et al. 2001).
Transgenic plants of cut rose, *R. hybrida* ‘Carefree Beauty’ were obtained using *A. tumefaciens* (Li et al. 2002). Three different explants including leaf, undifferentiated callus and embryogenic callus were used for transformation with *nptII* and *GUS* genes. Transgenic callus was obtained from both leaf and undifferentiated callus but neither developed into embryogenic callus. However, using embryogenic callus as explants produced transgenic secondary somatic embryos, which further developed into transgenic plants and flowered normally (Li et al. 2002).

Embryogenic callus of cut rose cultivars, *R. hybrida* ‘Only Love’ and ‘E006’ were used for transformation via *A. tumefaciens* (Condliffe et al. 2003). In this work was used, a binary vector system containing *nptII* and *GUS* genes alone or together with a ternary transformation system (Van der Fits et al. 2000). In addition, a VIP17 *ipt* construct containing *ipt* gene under the control of B4 and B5 domains of CaMV35S promoter was used in order to modify plant architecture. Transgenic plants were recovered via secondary somatic embryogenesis. Although, the ternary GUS construct produced greater transient expression than the ordinary system, results did not show significantly higher rates of stable GUS expression or plant recovery. In the experiment with *ipt* gene, since B4 and B5 domains of 35S promoter are specific to the base of the stem, *ipt* expression was moderate and transgenic lines were generally morphologically normal except for two lines which displayed abnormality including enlarged stems with an increased circumference and increased anthocyanin production. No change in leaf senescence retardation was reported in this study (Condliffe et al. 2003).

Transgenic plants of cut rose cultivar, *R. hybrida* 'Tineke’ were obtained via *A. tumefaciens* strains containing GFP reporter gene together with *nptII* gene (Kim et al. 2004). In addition, a plasmid containing *virE/virG* genes was also inserted into the same strain. Results showed that the selection of putative transgenic tissues could be based on GFP expression in rose and also showed that when additional copies of *virE* and *virG* genes were used, the number of putative transgenic callus was increased. Thus, these *vir* genes can improve the transformation efficiency in rose (Kim et al. 2004).

Despite its importance in flower industry, there are only these few available reports on transformation of rose cultivars. This is because of the recalcitrant nature of this genus like other woody plants. Most of the available protocols are also genotype dependent. Therefore, establishment of an efficient regeneration/ transformation protocol for transformation of each cultivar is necessary. However, except for *R. hybrida* ‘Moneyway’ which is a rootstock cultivar, all transformation studies on rose have been on either cut
rose or garden rose cultivars. To my knowledge, no report on transformation of miniature rose is available. In the present thesis, I report the successful transformation of miniature potted rose cultivar, *R. hybrida* ‘Linda’ (Manuscript).

Embryogenic callus containing somatic embryos were used for transformation via *A. tumefaciens* strains containing $P_{SAG12}$-IPT, $P_{SAG12}$-GUS or $P_{35S}$-GUSINT genes, all together with $nptII$ gene. The pSG529(+) and pSG514 plasmids (containing $P_{SAG12}$-IPT and $P_{SAG12}$-GUS, respectively) were received kindly from Prof. Richard M. Amasino (University of Wisconsin, Madison) and Prof. Susheng Gan (University of Cornell, New York). Transformation was successful and final results show that transgenic shoots could be obtained only from transformation with AGL1 strain when the inoculation culture was diluted in ½ MS medium. The presence of the transgene was confirmed with PCR, and Southern analysis revealed the existence of four different transgenic lines. Although, transgenic shoots showed difficulties in adventitious root induction, several shoots of one of the transgenic lines rooted and were transferred to the greenhouse for final analyses (Manuscript).
5. Somatic Embryogenesis Receptor-like Kinase (SERK) genes in different species

Since the process of somatic embryogenesis (see Chapter 2) is a genotype dependent and time-consuming event in some plants, several studies have been performed in recent years to find a reliable marker for distinguishing the competent cells in early stages of embryogenesis. Woody species like cacao (*Theobroma cacao*) and rose (*Rosa hybrida*) are recalcitrant in induction of somatic embryogenesis (Santos et al. 2005; Wann 1989; Zakizadeh et al., paper 2). During the transition from somatic to embryogenic state, differentiated cells will dedifferentiate first and then acquire the capacity for embryogenesis (Chapter 2). These cells are called competent cells and will turn into somatic embryos without the need for more treatments (Schmidt et al. 1997). These changes at the cellular level are the basis for finding a suitable marker.

5.1 Cellular changes during somatic embryogenesis

During somatic embryogenesis differentiated somatic cells change their fate towards developing to somatic embryos. During this transition, several changes occur in these cells including morphological, physiological, biochemical and gene expression changes. Among them, changes in cell morphology and gene expression have been used in some species as a marker for recognizing competent embryogenic cells from non-embryogenic somatic cells (Feher et al. 2003; Chugh and Khurana 2002).

5.1.1 Changes in cell morphology

Cell morphology has been considered as an early marker to distinguish cells having the competence to form embryos from non-embryogenic cells (Feher et al. 2003). A semi-automatic cell tracking method showed that in *Dactylis glomerata* (orchard grass) the competent cells are a subpopulation of small, isodiametric, cytoplasm-rich cells, even though a few percent of elongated, vacuolated cells also showed competence for embryogenesis (Somleva et al. 2000). Similar morphological traits were observed in alfalfa, where embryogenic cells were small, spherical and densely cytoplasmic, while non-embryogenic cells were elongated and highly vacuolated (Feher et al. 2003). However, in carrot (*Daucus carota*), using the same tracking system as for *Dactylis* indicated that the competent cells are highly variable in appearance and are indistinguishable from other cells according to their morphology (Schmidt et al. 1997; Toonen et al. 1994). Different morphology in embryogenically competent cells has also been observed in other species including chicory and *Picea abies* (Feher et al. 2003).
Since cell morphology did not provide a reliable marker to distinguish between embryogenic and non-embryogenic cells, the research continued to find a marker for this purpose.

5.1.2 Changes in gene expression

All morphological, physiological and biochemical changes which occur during somatic embryogenesis are the result of an alteration in gene expression (Santos et al. 2005). During somatic embryogenesis, some genes are expressed while others are repressed. These genes belong to various groups including signal transduction genes, homeotic genes, maturation genes and even housekeeping genes (reviewed by Chugh and Khurana 2002; Raghavan 1997). Figure 5-1 illustrates various genes participating in somatic embryogenesis (Chugh and Khurana 2002). Actin and tubulin which are usually used as internal control for expression studies, have been reported to exhibit higher expression during certain stages of somatic embryogenesis in some species due to enhanced cell wall and membrane formation (Chugh and Khurana 2002; Cyr et al. 1987). Since most of these genes are expressed at different stages of somatic embryos, they are not reliable markers for recognizing competent cells (Schmidt et al. 1997). Among the genes, which are expressed in early embryogenesis, until now only one type of gene has been shown to play a role in the acquisition of embryogenic competence in plant cells (Feher et al. 2003). This gene-type, which was discovered by Schmidt et al (1997) in carrot embryogenic cultures, exhibited homology with plant and animal receptor protein kinases, and was therefore designated as somatic embryogenesis receptor-like kinase (SERK) gene (Chugh and Khurana 2002; Feher et al. 2003). SERK genes from Daucus carota (DcSERK), Dactylis glomerata (DgSERK) and Arabidopsis thaliana (AtSERK1) have been used successfully as markers to distinguish competent embryogenic cells (Hecht et al. 2001; Schmidt et al. 1997; Somleva et al. 2000). Subsequently, several SERK genes have been isolated from different species and all of them belong to a large gene family known as the plant receptor-like kinase (RLK) superfamily.

5.2 Plant Receptor-like Kinase (RLK) gene superfamily

The plant receptor-like kinase (RLK) gene superfamily comprises a very large group of genes with at least 610 members in Arabidopsis (Shiu and Bleecker 2001; Tichtinsky et al. 2003). Among these, 417 genes encode RLK proteins which are characterised by an extracellular N-terminal domain, a single transmembrane spanning domain and a cytoplasmic C-terminal kinase domain with serine/threonine specificity (Cock et al. 2002; Ito et al. 2005; Shiu and Bleecker 2001; Tichtinsky et al. 2003). The other 193
genes are encoding protein kinases with no apparent signal peptide or transmembrane domain. These sequences are called receptor-like cytoplasmic kinases (RLCKs) and some of them might represent ancestral forms that were incorporated into receptor kinases by fusion of domains (Shiu and Bleecker 2001).

![Image](image1.png)

**Figure 5-1.** Different genes participating in somatic embryogenesis (Chugh and Khurana 2002).

Although the plant RLK family have some similarity to animal receptor kinases in some features such as certain mechanisms of down-regulation, presence of receptor complexes and receptor dimerization and transphosphorylation, there are clear differences between these two kingdoms and they can be grouped into separate monophyletic families (Cock et al. 2002; Tichinsky et al. 2003). The presence of ligand binding in several members of this large family and subsequent activation upon ligand binding are the reasons for the recent designation of these genes as plant receptor kinases (PRKs) (Thierry Gaude, personal communications).

Several studies have shown that RLK genes play a wide range of roles in plant growth and development and the plant defence system. These roles include cell and organ elongation, floral organ abscission, control of leaf development, epidermal cell
specification, self incompatibility (in Brassicaceae), maintenance of the apical meristem (in Arabidopsis), post meiotic pollen development, brassinosteroid signalling, nodulation, meristem development, bacterial resistance and plant defence responses (Cock et al. 2002; Shiu and Bleecker 2001; Tichtinsky et al. 2003). RLK proteins carry out these roles with transduction of extracellular signals to cytoplasmic kinase domains and propagate the signals by transphosphorylation of intracellular target proteins, which further transduce the signal to induce specific responses (Baudino et al. 2001; Ito et al. 2005).

Classification of RLK proteins, based on identity among extracellular domains, divided the 417 RLK proteins of Arabidopsis into more than 21 classes such as leucine-rich repeat (LRR)-RLK, S-domain RLK, L-lectin-RLK, RKF3-like-RLK and PERK-like-RLK (Fig. 5-2). Among these classes, LRR-RLK is the biggest one with 216 proteins in Arabidopsis. RLK proteins in this class contain various numbers of LRR in their receptor domains. Based on similarity among kinase domains, the LRR-RLK class further divided into 13 subfamilies called LRR I to LRR XIII. SERK proteins are members of LRR II subfamily (Ito et al. 2005; Shiu and Bleecker 2001).

5.3 Somatic Embryogenesis Receptor-like Kinase (SERK) family

The discovery of several SERK homologues from different plant species indicates the existence of a SERK gene family in plants. Some species have only one SERK
homologue while some others like *A. thaliana*, *Helianthus annuus* and *Zea mays* have several homologues of SERK genes which constitute their own SERK subfamily (Baudino et al. 2001; Hecht et al. 2001; Thomas et al. 2004). However, all SERK proteins have some similarities in their structures, which are used for their recognition.

5.3.1 Characteristics of the SERK family

In addition to the main characteristics of LRR-RLK proteins (signal peptide, LRR-domain, single transmembrane domain and cytoplasmic kinase domain with Ser/Thr specificity), all SERKs have proline-rich domains containing a Ser-Pro-Pro (SPP) motif between LRR and a transmembrane domain. In addition, most of them have a leucine-rich domain with a Leu zipper (LZ or ZIP) pattern between signal peptide and LRR domain. The SPP motif is unique for the SERK protein and considered as a hallmark of the SERK-like RLKs (Hecht et al. 2001; Schmidt et al. 1997). This motif contains the SPPPPP sequence that is also present in some plant cell wall proteins termed extensins (Schmidt et al. 1997). The SPP domain might have a role in providing flexibility to the extracellular domain or might interact with the cell wall (Hecht et al. 2001; Schmidt et al. 1997). As examples, SERKs from *Arabidopsis* (AtSERK1 and AtSERK2), maize (ZmSERK1 and ZmSERK2), rice (OsSERK1 and OsSERK2) and cacao (TcSERK) have both LZ and SPP domains in the extracellular parts (Baudino et al. 2001; Hecht et al. 2001; Ito et al. 2005; Santos et al. 2005), while SERKs from carrot (DcSERK) and *Citrus unshiu* (CitSERK1) have only the SPP motif (Schmidt et al. 1997; Shimada et al. 2005). Another similarity of the SERKs is that all of them have five repeats of LRR in their extracellular domains (Baudino et al. 2001; Hecht et al. 2001; Shimada et al. 2005).

5.3.2 SERK genes in different plant species

The first member of the SERK family in plants was reported in carrot (*Daucus carota*) by Schmidt et al. (1997). DcSERK is the only homologue of this gene family from this species. The next member was DgSERK, which was isolated from *Dactylis glomerata* (Somleva et al. 2000). Also from this species only one homologue has been reported until now. Since then, several SERK genes have been isolated from different plants. *A. thaliana* with five homologues (AtSERK1-5) has the highest number of SERK genes so far (Hecht et al. 2001). *Zea mays* with three homologues (ZmSERK1-3, Baudino et al. 2001), *Medicago truncatula* with one (MtSERK, Nolan et al. 2003), *Helianthus annuus* with four (HaSERK1-4, Thomas et al. 2004), *Theobroma cacao* with one (TcSERK, Santos et al. 2005), *Oryza sativa* with two (OsSERK1-2, Ito et al. 2005), *Poa pratensis*
with two \((PpSERK1-2, \text{Albertini et al. 2005})\) and \(Citrus\ unshiu\) with one \((CitSERK1, \text{Shimada et al. 2005})\) are some of the SERK genes reported in recent years.

For \(Rosa\ hybrida\ \text{cv. Linda}\), the isolation and characterisation of four SERK gene homologues have been described \((\text{Zakizadeh et al. 2007, paper 2})\). The genes were designated as \(RhSERK1-4\) (Appendix) and showed a high identity with other members of the SERK family, which were used in this study for designing degenerate primers. Accession numbers for some of the mentioned SERK genes are available in Table 5-1.

5.4 Identification of SERK genes in rose \((R.\ hybrida)\)

5.4.1 Isolation of novel SERK genes using degenerate primers

Degenerate primers can be used for isolating the genes encoding proteins that belong to known protein families \((\text{Rose et al. 1998})\) such as the SERK family in the present study. For designing degenerate primers, aligned homologues sequences of either proteins or nucleotides are used. In case of using protein sequences, primer design is based on reverse translation of multiply aligned sequences across the conserved regions of the proteins \((\text{Rose et al. 1998})\). The protein sequences corresponding to the target gene \((SERK\ in\ the\ present\ study)\) are obtained from GenBank (NCBI, TAIR or others) and may be aligned using Clustal W software \((\text{Thompson et al. 1994})\) available at \http://www.ebi.ac.uk/clustalw/\. Then, aligned sequences may be used for designing hybrid-degenerate primers with the CODEHOP program \((\text{Rose et al. 1998})\) available at \http://blocks.fhcrc.org/codehop.html\. Hybrid degenerate primers consist of a non-degenerate consensus part in the 5’ region and a relatively short degenerate part at the 3’ region. Reducing the length of the degenerate part \(\text{(low degeneracy)}\) will decrease the number of individual primers in the degenerate primer pool. This strategy, which has been used successfully for distantly related proteins, prevents mismatches between primers and template \((\text{Rose et al. 1998})\).

In the present study, SERK protein and nucleotide sequences from \(A.\ thaliana\ \((AtSERK1-5)\), carrot \((DcSERK)\), cacao \((TcSERK)\), \(Citrus\ unshiu\ \((CitSERK1)\) and \(Medicago\ truncatula\ \((MtSERK)\)\) were obtained from GenBank (NCBI and TAIR) and were aligned using Clustal W program. Four sets of degenerate primers were designed using CODEHOP program and manual method from aligned protein and nucleotide sequences, respectively. These degenerate primers were used in all different combinations for PCR screening of the \(R.\ hybrida\ \text{“Linda” genomic DNA} for SERK genes. Five of the combinations amplified fragments with the expected sizes. These
fragments were cloned and sequenced commercially and the sequences were used for identification of the fragments with bioinformatics tools (Zakizadeh et al. 2007, paper 2).

5.4.2 Characterisation of obtained sequences

5.4.2.1 BLAST

Obtained nucleotide sequences are submitted to the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) in order to compare the sequences with known sequences available in GenBank. BLAST, which can be used for finding members of a gene family as well as evolutionary relationship among genes (Altschul et al. 1990), have different choices including nucleotide blast, protein blast, blastx (for search protein database using a translated nucleotide query) and bl2seq (for aligning two protein or nucleotide sequences with each other) (Tatusova and Madden, 1999).

Table 5-1. Percent nucleotide identity between partially sequenced *Rosa hybrida* SERKs (*RhSERK1*–*RhSERK4*) and SERKs from *Arabidopsis thaliana* (*AtSERK1*-5), *Citrus unshiu* (*CitSERK1*), *Daucus carota* (*DcSERK*), cacao (*TcSERK*) and *Medicago truncatula* (*MtSERK*). (Accession numbers of *Arabidopsis* are from TAIR and the rest from NCBI GenBank).

<table>
<thead>
<tr>
<th></th>
<th>Acc. No.</th>
<th><em>RhSERK1</em></th>
<th><em>RhSERK2</em></th>
<th><em>RhSERK3</em></th>
<th><em>RhSERK4</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AtSERK1</em></td>
<td>2013020</td>
<td>81</td>
<td>78 – 79</td>
<td>76</td>
<td>73</td>
</tr>
<tr>
<td><em>AtSERK2</em></td>
<td>2026096</td>
<td>82</td>
<td>82 – 79</td>
<td>83</td>
<td>73</td>
</tr>
<tr>
<td><em>AtSERK3</em></td>
<td>2119259</td>
<td>80</td>
<td>83 – 84</td>
<td>83</td>
<td>73</td>
</tr>
<tr>
<td><em>AtSERK4</em></td>
<td>2040460</td>
<td>80</td>
<td>80 – 85</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td><em>AtSERK5</em></td>
<td>2040470</td>
<td>78</td>
<td>80 – 85</td>
<td>78</td>
<td>82</td>
</tr>
<tr>
<td><em>CitSERK1</em></td>
<td>AB115767</td>
<td>83</td>
<td>87 – 83</td>
<td>86</td>
<td>76</td>
</tr>
<tr>
<td><em>DcSERK</em></td>
<td>U93048</td>
<td>81</td>
<td>80 – 84</td>
<td>82</td>
<td>76</td>
</tr>
<tr>
<td><em>TcSERK</em></td>
<td>AY570507</td>
<td>84</td>
<td>90 – 84</td>
<td>89</td>
<td>78</td>
</tr>
<tr>
<td><em>MtSERK</em></td>
<td>AY162177</td>
<td>81</td>
<td>83 – 87</td>
<td>82 – 95</td>
<td>76</td>
</tr>
<tr>
<td><em>RhSERK1</em></td>
<td>EF631967</td>
<td>-</td>
<td>85</td>
<td>86</td>
<td>75</td>
</tr>
<tr>
<td><em>RhSERK2</em></td>
<td>EF631968</td>
<td>-</td>
<td>-</td>
<td>97</td>
<td>77</td>
</tr>
<tr>
<td><em>RhSERK3</em></td>
<td>EF631969</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td><em>RhSERK4</em></td>
<td>EF631970</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* The bl2seq alignments of *RhSERK2* with SERKs from other species identified 2 homologous regions corresponding to the 2 exon fragments in *RhSERK2* (see Fig.1, p89). The introns are not very conserved among species. The left exon-fragments of *RhSERK1*, 3 and 4 are only about 20 bp (Fig. 1, p89) and only for *RhSERK3/MtSERK* is a homology with this short fragment identified.

In the present study, nucleotide sequences obtained from sequencing were submitted to both nucleotide blast and blastx tools in NCBI. Results from both methods showed that fragments amplified from three primer combinations have high identity with SERK from
other species. These partial sequences were designated as RhSERK1- RhSERK4 and are available in NCBI GenBank with accession numbers EF631967-70 (Zakizadeh et al. 2007, paper 2). The bla2seq tool was also used to find identity among rose SERK sequences and also between each rose SERK sequence and each of SERK sequences which were used for designing degenerate primers, at both nucleotide (Table 5-1) and protein (Zakizadeh et al. 2007, paper 2- Table2) level.

5.4.2.2 Phylogenetic tree

A phylogenetic tree, which may also be called an evolutionary tree, demonstrates the evolutionary interrelationships among taxonomic groups derived from a common ancestor. After finding the identity of an unknown sequence and genes homologous to it in GenBank using BLAST, phylogenetic trees can be used to predict the genetic distance between a gene and each member of a corresponding gene family and members of other gene families. For example, SERK genes from maize (ZmSERK1 and ZmSERK2) show less genetic distance to DcSERK compared to SERK genes from Arabidopsis (AtSERK1-5) in a phylogenetic tree made with these species (Baudino et al. 2001). However, in a phylogenetic tree with more members of the SERK family, ZmSERK1 shows closer evolutionary relationship with SERK genes from Hordeum vulgare and Triticum aestivum than with ZmSERK2 (Santos et al. 2005). Phylogenetic trees can be made from either nucleotide or protein sequences. In any case, these sequences have to be aligned first and then the tree will be made using these aligned sequences.

In the present study, the phylogenetic tree was constructed by MegAlign (DNAsStar, Ver. 5.01) program using aligned SERK protein sequences from rose, Arabidopsis, carrot, cacao, Citrus and Medicago and the results showed that rose SERK proteins have more identity with SERK proteins from other species than with each other (Zakizadeh et al. 2007, paper 2). However, to see the evolutionary relationship of rose SERK genes among numerous genes from the SERK family, a bigger phylogenetic tree was constructed using several SERK genes. SERK nucleotide sequences from the above mentioned species in addition to sequences from maize (ZmSERK1-3), sunflower (HaSERK1-4), rice (OsSERK1) and Cyclamen persicum (CpSERK) were used for the construction of this tree (Fig 5-3). The tree shows that RhSERK4 has less genetic distance to OsSERK1 from a monocot than with other SERK genes from dicots used in this tree. This is in contrast to the results reported by Santos et al. (2005) where monocots and dicots were located in separate branches. RhSERK1 shows closer genetic relationship with AtSERK4 and AtSERK5. RhSERK2 and RhSERK3 are very similar (97% identity).
and located in the same branch. It may be suggested that these two sequences might be paralogous, which are results of a duplication event after specification.

Figure 5-3. Phylogenetic tree constructed using SERK genes from different species. Accession numbers are given in table 5-1. The accession numbers of genes not mentioned in table 5-1 are as follows: *CpSERK* (EF672247), *HaSERK1-4* (AF485384-87, respectively), *OsSERK1* (AB110224), *ZmSERK1-2* (AJ277702-3, respectively) and *ZmSERK3* (AJ400870).

5.5 Expression analysis of SERK genes

5.5.1 Expression studies in other species

Several studies have been performed on the expression of SERK genes before, during and after somatic embryogenesis in different species (Baudino et al. 2001; Hecht et al. 2001; Ito et al. 2005; Santos et al. 2005; Schmidt et al. 1997; Shimada et al. 2005; Somleva et al. 2000). SERK genes were first noticed when Schmidt et al. (1997) were looking for a reliable marker to be able to distinguish cells which are competent to produce somatic embryos from non-competent cells in carrot cell culture. Expression studies showed that *DcSERK* was expressed in some enlarged cells after 7 days of culture on 2,4-D enriched medium. These cells were derived from the provascular tissues. Untreated cells with 2,4-D did not express *SERK*. However, in established embryogenic carrot cell cultures, *DcSERK* was detected in cells with different morphology. *SERK*
expression was not restricted to competent single cells, but existed in small clusters of embryogenic cells. There was high level expression in small globular somatic embryos of up to about 100 cells. No SERK expression was observed during the mid to late globular, heart and torpedo stages of somatic embryogenesis. In carrot seeds, DcSERK was only expressed in early embryos up to the globular stages. No expression was seen in seedlings, roots, stems, leaves, developing or mature flower organs, pollen grains and stigmas before and after fertilization. SERK expression was not noted in parts of seeds such as seed coat, integuments, or embryo sac constituents before fertilization, and the endosperm at all stages of development, as well as the later stages of zygotic embryos (Schmidt et al. 1997). In Dactylis glomerata, the first SERK expressing cells were observed in younger parts of the leaves five days after culture initiation. Similar to carrot, they were mainly located close to the vascular bundles. DgSERK mRNA was present in preglobular 32-celled embryos and was not detectable later on in embryos of the transition stage. Neither explant cells nor developing somatic embryos showed SERK expression. In contrast to carrot where SERK was not expressed in somatic embryos after the globular stage, the DgSERK gene was expressed continuously in the shoot apical meristem and transiently in the protoderm, coleoptile and coleorhiza (Somleva et al. 2000). In A. thaliana, AtSERK1 was highly expressed in cells acquiring embryogenic competence, in embryogenic cells and in early somatic embryos up to the heart stage. After this stage, AtSERK1 transcripts were not detected in the later stages of embryo development (Hecht et al. 2001). Therefore, similar to DcSERK and DgSERK, AtSERK1 can be used as a marker for finding competent cells in the culture. In zygotic embryogenesis, AtSERK1 mRNA was most abundant in ovules before fertilization and transiently during zygotic embryo development. Low expression was also found in vascular tissue. Transgenic lines of Arabidopsis plants obtained after transformation with P35S-AtSERK1 gene, exhibited 3- to 4-fold increase in efficiency for initiation of somatic embryogenesis. These results confirmed that AtSERK1 could confer embryogenic competence to the culture (Hecht et al. 2001).

Except for DcSERK, DgSERK and AtSERK1, which have been used successfully as markers for embryogenic capacity, other SERK genes exhibited various expression patterns in different tissues, which make them difficult to use as markers. In rice, expression analysis showed that OsSERK1 was expressed with different levels in all organs including flowers one day after pollination, panicle, leaf blade, leaf sheath, root, shoot apex and in callus on either callus induction medium or on regeneration medium. The OsSERK2 gene was also expressed in all organs at similar levels (Ito et al. 2005).
maize, while expression of ZmSERK2 was relatively uniform in all investigated samples (embryogenic and non-embryogenic callus cultures, somatic embryos at different stages, mature leaves and root tips), expression of ZmSERK1 was neither detected in non-reproductive tissues like mature leaf and root tips, nor in somatic embryos. The ZmSERK1 was expressed more in male and female reproductive tissues with high expression in microspores (Baudino et al. 2001). In Citrus unshiu, transcripts of CitSERK1 were observed in embryogenic cultures, which started embryo formation up to the plantlet stage, and were not detected in non-embryogenic proliferating callus. However, CitSERK1 was expressed in all tissues including flowers, stem, leaves and fruits at 30 days after flowering (DAF) and 60 DAF, but not at 180 DAF (Shimada et al. 2005). Expression of MtSERK1 from M. truncatula was doubled in conditions favouring callus and somatic embryos compared to conditions favouring callus and root formation. However, expression of MtSERK1 in non-embryogenic tissues suggests that this gene may have a broader role in morphogenesis rather than being limited to somatic embryogenesis (Nolan et al. 2003). And finally in cacao, high expression of TcSERK was detected in initially induced embryogenic callus and in repetitive embryogenic callus and a weak signal was found in somatic and zygotic embryos and leaves and no transcript was observed in roots, petals and staminoids (Santos et al. 2005). Therefore, although the expression of TcSERK in leaves indicated the non-specificity of this gene to somatic embryogenesis, it is more reliable as a SERK marker than SERK genes from rice, maize, Citrus and M. truncatula.

5.5.2 Expression studies in rose

In Rosa hybrida “Linda” expression analysis of RhSERK genes showed that all four rose SERKs were expressed with various levels in both embryogenic and non-embryogenic tissues including petals, stamen, leaves, non-embryogenic callus, embryogenic callus, mature embryos and roots. Expression in embryogenic cultures suggests a role for SERK genes in somatic embryogenesis; however, expression in non-embryogenic cultures indicates a broader function of these genes in this species. (Zakizadeh et al. 2007, paper 2). Therefore, similar to SERK genes from rice, maize, Citrus and M. truncatula, RhSERK1-4 are not reliable markers for embryogenic capacity.

In conclusion, expression studies in the above mentioned species indicate a broader role of SERK genes in plant growth and development rather than specific function(s) in somatic embryogenesis. Thus, in some of the species SERK genes cannot be used as trustable markers.
Publications
Paper I

Regeneration of miniature potted rose (*Rosa hybrida* L.) via somatic embryogenesis

Hedayat Zakizadeh, Thomas Debener, Sridevy Sriskandarajah, Stefan Frello & Margrethe Serek

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Regeneration of Miniature Potted Rose (Rosa hybrida L.) via Somatic Embryogenesis

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Summary

Flowering plants were regenerated from in vitro grown leaves of miniature rose (Rosa hybrida L. ‘Linda’) via somatic embryogenesis. Sufficient amounts of callus were produced on in vitro leaves of 8 cultivars ('Leonie', 'Linda', 'Sonja', 'Toledo', 'Tiffany', 'Mette', 'Ema' and 'Andromeda') incubated on MS media fortified with either 45.2 or 90.5 µM 2,4- dichlorophenoxyacetic acid (2,4-D) for 6 weeks. Except for 'Tiffany' and 'Andromeda', embryogenic callus was induced on the callus of all cultivars incubated on MS media supplemented with different concentrations of zeatin or thidiazuron (TDZ). The highest frequency of primary somatic embryogenesis (30 %) was achieved with 'Sonja' on medium containing 22.7 µM TDZ, but the embryos from 'Sonja' failed to develop into plants. Only the somatic embryos of 'Linda' developed into flowering plants thus further work was carried out with this cultivar. The frequency of primary somatic embryogenesis in 'Linda' was 3.3 % on medium containing either 45.6 µM zeatin or 45.4 µM TDZ. The highest proliferation rate (3.8-fold) and the highest frequency of embryo maturation (76.6 %) were obtained on 1/2 MS medium fortified with 7.57 µM abscisic acid. Germination and subsequent shoot development on mature somatic embryos occurred in a medium containing 1.42 µM 1-naphthaleneacetic acid (NAA), 17.76 µM 6-benzylaminopurine (BAP) and 1.44 µM gibberellic acid (GA3). Shoots were rooted on 1/2 MS medium supplemented with 5.7 µM 3- indole acetic acid (IAA) and 9.84 µM indole-3- butyric acid (IBA) and transferred to the greenhouse. The plants flowered after about 10 weeks and were morphologically indistinguishable from the original plant.

Key words. callus – plant growth regulator – plant regeneration – rose – somatic embryos

Introduction

Rose, the economically most important ornamental plant worldwide, is cultivated widely for use as garden plants, cut flowers, potted plants and for the perfume industry. Production of new cultivars is driven by grower and customer demands for new traits such as pest and disease resistance, longer vase life, new flower forms, color and scent. Conventional breeding methods for introducing new genes into rose cultivars are constrained by a limited gene pool and a high degree of inter-cultivar sterility due to different ploidy levels. The process is also time-consuming. Biotechnological methods, such as biolistic or Agrobacterium-mediated transformation, could provide an easy way to introduce a desired gene into established cultivars.

The main prerequisite for genetic transformation is whole plant regeneration from transformable explants. Although the frequency of somatic embryogenesis in rose is generally low and the maximum reported is 37 percent, several reports have concluded that somatic embryos are the best material for transformation of this plant species (ROUT et al. 1991; Firoozabady et al. 1994; VAN DER SALM et al. 1996; CONDLIFFE et al. 2003).

There is not any universal, genotype-independent method for somatic embryogenesis in rose. Different explants including in vitro grown leaves (VISSESSUWAN et al. 1997; DOHIN et al. 2001), immature leaves and internodal stem segments (ROUT et al. 1991; HSIA and KORBAN 1996; LI et al. 2002), greenhouse grown leaves (KITZIOS et al. 1999), in vitro derived petioles and roots (MARCHANT et al. 1996) and adventitious roots (DERKS et al. 1995; VAN DER SALM et al. 1996), petals (MURALI et al. 1996), filaments (NORIEGA and SONDHAHL 1991) and different flower parts (ARENE et al. 1993) have been used for induction of somatic embryogenesis in different rose cultivars. Although embryogenic callus were derived in some rose cultivars, not all studies resulted in the successful recovery of flowering plants. Also, except for R. chinensis minima ‘Red Sunblaze’ and ‘Baby Katie’ (HSIA and KORBAN 1996; LI et al. 2002) which are miniature roses, the rest of the investigated cultivars were either garden roses or cut roses or used as both. Denmark is the largest producer of miniature potted roses in the world. Therefore, the present study investigated whole plant regeneration via somatic embryogenesis of eight miniature potted rose cultivars which are commercially grown in Denmark, in order to proceed with genetic transformation.
Materials and Methods
Establishment of shoot cultures

Proliferating shoot cultures of 8 miniature rose cultivars (‘Leonie’, ‘Linda’, ‘Sonja’, ‘Toledo’, ‘Tiffany’, ‘Mette’, ‘Etta’ and ‘Andromeda’) were established *in vitro* by surface sterilized nodal stem segments of greenhouse grown plants. Sterilization was carried out using 2 % sodium hypochlorite and 0.1 % tween-20 in screw-capped bottles for 20 min with vigorous shaking every 5 min followed by rinsing 3–5 times with sterile deionized water. For ‘Etta’, 3 % sodium hypochlorite was necessary for sterilization due to its compact growth habit. Sterilized nodal stem segments were cultured in plastic containers (99 mm × 91 mm, Danefeld, Research and Breeding Station, Denmark) containing full strength MS (MURASHIGE and SKOOG 1962) salts supplemented with 0.021 μM NAA, 4.44 μM BAP, 0.28 μM GA3 and solidified with 4 g l⁻¹ agar (Bacto Agar, Becton, Dickinson and Company, USA) and 1.5 g l⁻¹ Gelrite (Duchefa, The Netherlands). The shoots were cultured in the above mentioned medium, but without GA3 every 6 weeks.

All media used in the study consisted of half- or full-strength MS salts, Staba vitamins (STABA 1969), 100 mg l⁻¹ myoinositol, 20 g l⁻¹ sucrose and 10 g l⁻¹ glucose. However, for shoot proliferation and elongation medium, FeEDTA (which is part of the standard MS salts) was replaced by 96 mg l⁻¹ FeEDDHA (Duchefa, The Netherlands), a more stable iron chelate. The pH of all media were adjusted to 5.7 using 1 N NaOH and either 8 g l⁻¹ agar or 3 g l⁻¹ Gelrite or a combination of both gelling agents (4 g l⁻¹ agar and 1.5 g l⁻¹ Gelrite) was added prior to autoclaving at 121 °C for 20 min.

Production of callus on in vitro grown leaves

Various growth regulators selected on the basis of previous reports (ROUT et al. 1991; HSIA and KORBAN 1996; KENTZIOS et al. 1999; DOHM et al. 2001; LI et al. 2002) were used primarily to induce sufficient callus on in vitro grown leaves of these cultivars for further experiments. Five different concentrations of 2,4-D (Table 1) were selected after obtaining promising results from preliminary experiments. Whole leaves of each cultivar were excised with a scalpel. Leaves were placed on the medium in 90 × 15 mm Petri dishes with abaxial side down. Media consisted of MS salts and different concentrations of 2,4-D (4.52, 13.5, 45.2, 90.5 and 135.7 μM) and solidified with 3 g l⁻¹ Gelrite. All treatments were repeated three times and each replication consisted of a single Petri dish containing 10 leaf explants. All cultures were kept in the dark at 23±2°C for about six weeks and then data on numbers of explants with non-embryogenic and embryogenic callus were recorded.

Induction of primary somatic embryos

Callus produced on media containing either 45.2 or 90.5 μM 2,4-D (Table 1) was transferred to new media for induction of somatic embryos. Explants with few callus and the ones with excessive callus (produced on 135.7 μM 2,4-D) were also transferred to embryo induction media separately, but the results were not used for data analysis. At this stage three sub experiments were carried out: In the first, explants of all cultivars (Table 1) with sufficient callus were incubated on MS medium, supplemented with various concentration of zeatin (18.2, 27.4, 45.6 and 91.2 μM) or thidiazuron (TDZ, 4.5, 13.6, 22.7 and 45.4 μM) and solidified with 8 g l⁻¹ agar. Explants were subcultured on fresh medium once every month for five to seven months (depending on the cultivar). In the second sub experiment, calluses from ‘Linda’ and ‘Toledo’ were placed on the same media as above. Following two consecutive passages on fresh media, cal- lus was transferred to the plant growth regulator- (PGR) free media. In the third sub experiment, callus from ‘Linda’ and ‘Leonie’ was incubated on 1/2 MS medium with the same concentration of zeatin and TDZ as above. Following three consecutive passages, explants were transferred to PGR-free media.

All treatments were repeated three times and each replication consisted of a single Petri dish containing 10 callus explants. The number of explants that produced embryogenic callus was recorded.

Proliferation and maturation of primary somatic embryos

The most promising cultivars, ‘Linda’ and ‘Sonja’, were chosen for the subsequent experiments. Hard and compact embryogenic callus of ‘Linda’ and friable embryogenic callus of ‘Sonja’ from the first experiments were transferred to a new medium for proliferation and maturation of somatic embryos. This medium consisted of 1/2 MS salts, solidified with 8 g l⁻¹ agar and supplemented with four levels of ABA (3.78, 7.57, 15.13 and 31.30 μM) and solidified with 8 g l⁻¹ Gelrite. Explants were subcultured on fresh medium once every month for five to seven months (depending on the cultivar). In the second sub experiment, calluses from ‘Linda’ and ‘Toledo’ were placed on the same media as above. Following two consecutive passages on fresh media, callus was transferred to the plant growth regulator- (PGR) free media. In the third sub experiment, callus from ‘Linda’ and ‘Leonie’ was incubated on 1/2 MS medium with the same concentration of zeatin and TDZ as above. Following three consecutive passages, explants were transferred to PGR-free media.

All treatments were repeated three times and each replication consisted of a single Petri dish containing 10 callus explants. The number of explants that produced embryogenic callus was recorded.

### Table 1. Effect of different concentrations of 2,4-D on callus production in leaf explants of 8 miniature rose cultivars (*Rosa hybrida*).

<table>
<thead>
<tr>
<th>2,4-D (μM)</th>
<th>No. of leaf explants</th>
<th>‘Linda’</th>
<th>‘Leonie’</th>
<th>‘Sonja’</th>
<th>‘Toledo’</th>
<th>‘Tiffany’</th>
<th>‘Mette’</th>
<th>‘Andromeda’</th>
<th>‘Etta’</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.52</td>
<td>30</td>
<td>2.0 d</td>
<td>5.3 c</td>
<td>1.7 c</td>
<td>2.7 b</td>
<td>4.0 c</td>
<td>7.7 c</td>
<td>1.0 c</td>
<td>16.0 c</td>
</tr>
<tr>
<td>13.5</td>
<td>30</td>
<td>36.0 c</td>
<td>36.0 b</td>
<td>53.0 b</td>
<td>18.7 b</td>
<td>60.0 b</td>
<td>62.0 b</td>
<td>51.6 b</td>
<td>61.0 b</td>
</tr>
<tr>
<td>45.2</td>
<td>30</td>
<td>88.6 b</td>
<td>88.3 a</td>
<td>100.0 a</td>
<td>72.0 a</td>
<td>91.0 a</td>
<td>88.7 ab</td>
<td>96.6 a</td>
<td>86.3 a</td>
</tr>
<tr>
<td>90.5</td>
<td>30</td>
<td>97.0 ab</td>
<td>97.0 a</td>
<td>90.0 a</td>
<td>82.3 a</td>
<td>100.0 a</td>
<td>94.7 ab</td>
<td>98.3 a</td>
<td>87.3 a</td>
</tr>
<tr>
<td>135.7</td>
<td>30</td>
<td>99.0 a</td>
<td>100.0 a</td>
<td>98.0 a</td>
<td>85.3 a</td>
<td>99.0 a</td>
<td>99.3 a</td>
<td>100.0 a</td>
<td>93.3 a</td>
</tr>
</tbody>
</table>

*Means with different letter in each column are significantly different at P<0.05.*
Fig. 1. Plant regeneration from somatic embryos derived from in vitro leaves of miniature rose. Rosa hybrida ‘Linda’. (A) Embryogenic callus together with somatic embryos induced on callus produced on 2,4-D (x1). (B) Friable embryogenic callus with malformed somatic embryos (x9). a) One somatic embryo with several cotyledons b) One somatic embryo with one cotyleden. (C) Cluster of mature somatic embryos with cotyledonary leaves (x9). (D) Greening and germination (arrows) of mature somatic embryos one month after transferring to regeneration media (x4). (E) Abnormal shoot regenerated from germinated embryos and remained undeveloped (x4). (F) Regeneration and elongation of normal shoot (arrow) from germinated somatic embryos (x1). (G) In vitro adventitious root production on multiplied shoots (x0.5). (H) Plantlet two weeks after transferring to soil (x0.5) and (I) flowering plant 2 months after transferring to 9 cm plastic pot (x0.3).

18.92 µM). These concentrations were selected according to results of previous publications (LI et al. 2002; SHARMA et al. 2004; OGATA et al. 2005) and our preliminary experiments. Each treatment was replicated three times and each replication consisted of a single Petri dish containing 1 g embryogenic callus (10 callus clumps, 100 mg each). Data on increased weight of embryogenic callus (proliferation rate) and also numbers of explants with mature embryos (embryos with developed cotyledons) were collected after 6 weeks.

Development of somatic embryos into plants

Since somatic embryos from ‘Sonja’ did not develop into normal plants in preliminary experiments, further work was continued with ‘Linda’. Mature somatic embryos of ‘Linda’ (Fig. 1C) were transferred to media with MS salts and FeEDDHA iron chelate, supplemented with different concentrations of NAA (0, 1.42 µM), BAP (0, 4.4, 8.9, 17.7, 26.6 µM) and GA3 (0, 0.29, 1.44 µM) in various combinations (Table 4) for germination and shoot development. All media were solidified with 4 g l⁻¹ agar and 1.5 g l⁻¹ Gelrite. Specific hormonal concentrations used in these media were selected according to previous publications (ROUT et al. 1991; MARCHANT et al. 1996; VISESSUWAN et al. 1997; KINTZIOS et al. 1999) and the results from preliminary experiments of the present study. Mature somatic embryos were subcultured in fresh medium after one month and then regenerated shoots were transferred to the shoot multiplication medium containing 0.021 µM NAA and 4.44 µM BAP. Each treatment was repeated 3 times and each replication consisted of a single Petri dish (90 × 18 mm) containing 10 explants (Table 4). Data on the number of explants with normal and abnormal regenerated shoots were collected.

Results and Discussion

Callus production

Varying amounts of callus were produced on adaxial side of the leaves 6 weeks after incubation on different 2,4-D concentrations (Table 1). Both genotype and 2,4-D concentrations influenced the explants’ ability to form callus. 2,4-D at 4.52 µM produced very little callus on all cultivars tested in the present study, but this concentration produced sufficient callus in garden roses, R. hybrida ‘Pariser Charme’ and ‘Heckenzauber’ (DOHM et al. 2001).

Leaves of miniature roses became necrotic and died after two weeks of incubation on this 2,4-D concentration. Although 13.5 µM 2,4-D produced callus in all cultivars tested in the present study, but this concentration produced sufficient callus in garden roses, R. hybrida ‘Pariser Charme’ and ‘Heckenzauber’ (DOHM et al. 2001). Leaves of miniature roses became necrotic and died after two weeks of incubation on this 2,4-D concentration. Although no significant difference was observed between 45.2 and 90.5 µM 2,4-D in percentage of callus production on ‘Lin-
Table 2. Effect of cytokinins (Zeatin and TDZ) on induction of embryogenic callus in callus explants of 8 miniature rose cultivars.

<table>
<thead>
<tr>
<th>Zeatin (µM)</th>
<th>TDZ (µM)</th>
<th>No. of explants</th>
<th>'Linda'</th>
<th>'Leonie'</th>
<th>'Sonja'</th>
<th>'Toledo'</th>
<th>'Tiffany'</th>
<th>'Mette'</th>
<th>'Andromeda'</th>
<th>'Etna'</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.2</td>
<td>0.0</td>
<td>30</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>13.3 ab</td>
<td>3.3 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 b</td>
</tr>
<tr>
<td>27.4</td>
<td>0.0</td>
<td>30</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>13.3 ab</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 b</td>
</tr>
<tr>
<td>45.6</td>
<td>0.0</td>
<td>30</td>
<td>3.3 a</td>
<td>0.0 a</td>
<td>10.0 ab</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 b</td>
</tr>
<tr>
<td>91.2</td>
<td>0.0</td>
<td>30</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 b</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 b</td>
</tr>
<tr>
<td>0.0</td>
<td>4.5</td>
<td>30</td>
<td>2.3 a</td>
<td>3.3 a</td>
<td>6.7 ab</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>3.3 a</td>
<td>0.0 a</td>
<td>0.0 b</td>
</tr>
<tr>
<td>0.0</td>
<td>13.6</td>
<td>30</td>
<td>2.3 a</td>
<td>3.3 a</td>
<td>6.7 ab</td>
<td>3.3 a</td>
<td>0.0 a</td>
<td>6.7 a</td>
<td>0.0 a</td>
<td>0.0 b</td>
</tr>
<tr>
<td>0.0</td>
<td>22.7</td>
<td>30</td>
<td>0.0 a</td>
<td>3.3 a</td>
<td>30.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>3.3 b</td>
</tr>
<tr>
<td>0.0</td>
<td>45.4</td>
<td>30</td>
<td>0.0 a</td>
<td>3.3 a</td>
<td>3.3 a</td>
<td>10.0 ab</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>16.7 a</td>
</tr>
</tbody>
</table>

Means with different letter in each column are significantly different at P<0.05.

d'a', 'Leonie', 'Toledo', 'Mette' and 'Etna', the amount of callus in each explant produced by 45.2 µM 2,4-D was less than 200 mg explant⁻¹ in the follow-up experiment. Therefore 90.5 µM 2,4-D was used in further experiments in these cultivars. Although 135.7 µM 2,4-D also produced sufficient callus (more than 700 mg explant⁻¹) in all cultivars, the callus was too fragile to subculture and was not used in further experiments. In contrast to our results, increased concentrations of 2,4-D have reduced callus production in some rose cultivars (Li et al. 2002; Van der Salm et al. 1996).

Low concentrations of auxin (2,4-D, NAA, IBA, dicamba or p-chlorophenoxyacetic acid), alone or together with a cytokinin (BAP, kinetin, zeatin or 2iP), have been used successfully for callus production in rose cultivars (Noriega and Söndahl 1991; Rout et al. 1991; Arene et al. 1993; Deeks et al. 1995; Hsia and Korbán 1996; Marchant et al. 1996; Murali et al. 1996; Van der Salm et al. 1996; Kintzios et al. 1999; Dohm et al. 2001) but only high concentrations of 2,4-D produced sufficient callus in cultivars tested in the present study. However, most investigated cultivars were garden roses, which have a higher growth rate than miniature roses. The slow growth rate of miniature roses might be due to low endogenous hormonal levels, and this nature may create a demand for a high exogenous auxin concentration for callus production. Callus production in miniature rose, R. chinensis minima 'Red Sunblaze', which is the ancestor for all miniature roses (Horn 1992), also required 45.2 µM 2,4-D, a concentration that is considered high (Li et al. 2002). However, by using high auxin concentrations in callus production stage, somatic embryogenesis was reduced in some (Hsia and Korbán 1996), while increased in other rose cultivars (Marchant et al. 1996; Visesuwann et al. 1997).

Cultivars and treatments had an influence on the colour and friability of the callus. As the concentration of 2,4-D was raised, the friability of the callus markedly increased. Calluses from 'Linda', 'Leonie' and 'Toledo' were compact and pale-green in colour, while callus initiated from 'Sonja' was soft, friable and yellowish in colour. Calluses from 'Etna' and 'Mette' were compact and brown. Embryogenic callus was not observed at callus production stage in any of these cultivars, but rhizogenic calluses were produced with different frequencies in some cultivars (data not shown). 'Sonja', 'Etna', 'Mette' and 'Andromeda' produced rhizogenic callus and among them 'Mette' showed the highest frequency of rhizogenic callus, whereas rhizogenic callus was not observed on 'Linda', 'Leonie', 'Tiffany' and 'Toledo'.

Induction of primary somatic embryogenesis

Explants with sufficient callus produced on certain 2,4-D concentrations from each cultivar (according to above mentioned results) were transferred to MS medium containing zeatin or TDZ and sub-cultured once every month. In the first sub experiment, embryogenic callus (Fig. 1A) was observed after 2 months in 'Toledo' and 'Sonja', after 3 months in 'Linda' and 'Leonie' and after 4 months in 'Etna' and 'Mette'. Repeated subculture on fresh medium increased embryogenesis in 'Sonja' and 'Leonie' but not in other cultivars. No embryogenic callus was observed in 'Tiffany' and 'Andromeda' after 5 subcultures on induction medium. The percentage of embryogenic callus induction for most of the cultivars was about 3.3. However, 'Sonja' showed a significant response to reach 30 % embryogenic callus induction (Table 2). The highest percentages of embryogenic callus in 'Sonja' (30 %) and 'Etna' (16.7 %) were induced on media containing 22.7 and 45.4 µM TDZ, respectively. Except for these two cultivars, no significant difference was observed between various concentrations of zeatin and TDZ in induction of somatic embryogenesis. In general, zeatin induced embryogenesis in 3 cultivars, while TDZ did so in 6 cultivars. Also, the highest embryogenesis percentage was obtained by using 13.6 and 45.4 µM TDZ. The reason for better response by using TDZ might be due to the higher stability of TDZ in plant tissues compared with that of zeatin (Mok et al. 2000). Li et al. (2002) also obtained somatic embryogenesis in miniature rose, R. chinensis minima 'Carefree Beauty' by using TDZ concentrations ranging from 2.3 to 54.5 µM, with and without 2.9 µM GA3.

Various PGRs have been used for inducing embryogenic callus in different species and cultivars (reviewed by Jannes et al. 2005). Different concentrations of NAA (0.27 to 26.8 µM) alone or together with a cytokinin (such as kinetin, zeatin, BAP or TDZ) and/or GA3 have been used successfully for somatic embryogenesis in some rose cultivars (Noriega and Söndahl 1991; Rout et al. 1991; Arene et al. 2002). However, by using high auxin concentrations in callus production stage, somatic embryogenesis was reduced in some (Hsia and Korbán 1996), while increased in other rose cultivars (Marchant et al. 1996; Visesuwann et al. 1997).

**Table 2. Effect of cytokinins (Zeatin and TDZ) on induction of embryogenic callus in callus explants of 8 miniature rose cultivars.**

- Zeatin (µM): 18.2, 27.4, 45.6, 91.2, 0.0, 0.0, 0.0, 0.0
- TDZ (µM): 0.0, 0.0, 0.0, 0.0, 4.5, 13.6, 22.7, 45.4
- No. of explants: 30, 30, 30, 30, 30, 30, 30, 30
- Cultivars: 'Linda', 'Leonie', 'Sonja', 'Toledo', 'Tiffany', 'Mette', 'Andromeda', 'Etna'
- Percent of callus explants forming embryogenic callus:
  - 'Linda': 0.0 a, 0.0 a, 13.3 ab, 3.3 a, 0.0 a, 0.0 a, 0.0 a, 0.0 b
  - 'Leonie': 0.0 a, 0.0 a, 13.3 ab, 0.0 a, 0.0 a, 0.0 a, 0.0 a, 0.0 b
  - 'Sonja': 0.0 a, 3.3 a, 6.7 ab, 0.0 a, 0.0 a, 3.3 a, 0.0 a, 0.0 b
  - 'Toledo': 0.0 a, 0.0 a, 6.7 ab, 3.3 a, 0.0 a, 3.3 a, 0.0 a, 0.0 b
  - 'Tiffany': 0.0 a, 0.0 a, 3.3 a, 0.0 a, 0.0 a, 3.3 a, 0.0 a, 0.0 b
  - 'Mette': 0.0 a, 0.0 a, 3.3 a, 0.0 a, 0.0 a, 3.3 a, 0.0 a, 0.0 b
  - 'Andromeda': 0.0 a, 0.0 a, 3.3 a, 0.0 a, 0.0 a, 3.3 a, 0.0 a, 0.0 b
  - 'Etna': 0.0 a, 0.0 a, 3.3 a, 10.0 ab, 0.0 a, 0.0 a, 0.0 a, 16.7 a

Means with different letter in each column are significantly different at P<0.05.
In general, induction of embryogenic callus from vegetative explants is driven by an auxin or by a combination of auxin and cytokinin (RAEMAKERS et al. 1995). In contrast to MARCHANT et al. (1996), who obtained up to 36% embryogenic callus in *R. hybrida* 'Trumpeter' and 'Glad Tidings' after 2–4 months of repeated subculture on 13.5 \( \mu \)M 2,4-D, we did not detect any visible embryogenic callus with different 2,4-D concentrations. However, we obtained embryogenic callus in media supplemented with only cytokinin. Since we used high concentrations of 2,4-D in callus production stage it is possible that the embryos were induced by 2,4-D, but the proembryogenic cell masses did not develop into an easy visible stage. In addition, a carry over effect of this hormone might have had a role on induction of embryogenic callus together with cytokinin. Nevertheless, the use of TDZ alone or together with GA3 resulted in a high frequency of somatic embryogenesis in *R. hybrida* 'Carefree Beauty' (HAIR and KORBAN 1996; LI et al. 2002). In contrast to these results, low levels of TDZ inhibited somatic embryogenesis in *R. hybrida* 'Carl Red' (VISESSUWAN et al. 1997).

Three types of embryogenic callus were observed in the present study: hard and compact callus with a yellow colour, which was produced in 'Linda' and 'Toledo'; friable jelly-like yellowish callus in 'Sonja' and the same texture but white in 'Leonie', 'Etna' and 'Mette'. Friable white callus also initiated from compact callus of 'Linda' upon subculture on the embryogenic callus proliferation medium. Proliferation of friable embryogenic callus in preliminary experiments resulted in high numbers of abnormal somatic embryos with one or more than two cotyledons (Fig. 1B). Therefore, this type of embryogenic callus was not used for further experiments. As indicated by VISESSUWAN et al. (1997), high moisture content in friable embryogenic callus appears to be responsible for producing embryos with undeveloped apical meristems. High concentrations of cytokinins can also lead to formation of abnormal somatic embryos. In some species, use of 2,4-D resulted in a high frequency of morphologically abnormal embryos which failed to convert into plants (JIMENEZ 2005). However, abnormality of somatic embryos is common in many species (DODEMAN et al. 1997) and it has also been reported in many rose cultivars.

Embryogenic callus was not obtained from the second and third sub experiments in the present study, whereas PGR-free media previously have been used successfully for somatic embryogenesis in some rose cultivars (KUNITAKE et al. 1993; DERKS et al. 1995; VAN DER SALM et al. 1996; VISESSUWAN et al. 1997).

### Table 3. Effect of different ABA concentrations on proliferation rate of embryogenic callus (increased weight of embryogenic callus after 6 weeks) and maturation of somatic embryos in 'Linda' and 'Sonja'.

<table>
<thead>
<tr>
<th>ABA (( \mu )M)</th>
<th>Proliferation rate of embryogenic callus (fold)</th>
<th>Percent embryo maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'Linda'</td>
<td>'Sonja'</td>
</tr>
<tr>
<td>3.78</td>
<td>3.76 a</td>
<td>2.10 ab</td>
</tr>
<tr>
<td>7.57</td>
<td>3.86 a</td>
<td>1.16 b</td>
</tr>
<tr>
<td>15.13</td>
<td>3.30 a</td>
<td>3.97 a</td>
</tr>
<tr>
<td>18.92</td>
<td>3.10 a</td>
<td>1.87 ab</td>
</tr>
</tbody>
</table>

Means with different letter in each column are significantly different at P<0.05.

### Table 4. Effects of different media on development of somatic embryos in 'Linda' cultivar.

<table>
<thead>
<tr>
<th>Media</th>
<th>Growth regulators</th>
<th>No. of callus clumps</th>
<th>Percent embryo regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAA (( \mu )M)</td>
<td>BAP (( \mu )M)</td>
<td>GA3 (( \mu )M)</td>
</tr>
<tr>
<td>P</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>A</td>
<td>1.42</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>B</td>
<td>1.42</td>
<td>4.44</td>
<td>0.29</td>
</tr>
<tr>
<td>C</td>
<td>1.42</td>
<td>4.44</td>
<td>1.44</td>
</tr>
<tr>
<td>D</td>
<td>1.42</td>
<td>8.90</td>
<td>0.29</td>
</tr>
<tr>
<td>E</td>
<td>1.42</td>
<td>8.90</td>
<td>1.44</td>
</tr>
<tr>
<td>F</td>
<td>1.42</td>
<td>17.76</td>
<td>0.29</td>
</tr>
<tr>
<td>H</td>
<td>1.42</td>
<td>17.76</td>
<td>1.44</td>
</tr>
<tr>
<td>I</td>
<td>1.42</td>
<td>26.60</td>
<td>0.29</td>
</tr>
<tr>
<td>J</td>
<td>1.42</td>
<td>26.60</td>
<td>1.44</td>
</tr>
</tbody>
</table>

Means with different letter in each column are significantly different at P<0.05.
For quantification, “proliferation rate” was used which represents the ratio of the fresh weight of the embryogenic callus together with somatic embryos generated at the end of 6 weeks of incubation on ABA, to the initial fresh weight of primary embryogenic callus inoculated. In ‘Linda’ 7.57 µM ABA produced the highest proliferation rate (3.8-fold, Table 3), although there was no statistically proven effect of ABA concentration. In ‘Sonja’, 15.13 µM ABA encouraged higher proliferation of embryogenic callus (3.9-fold) than other concentrations (Table 3). The number of callus clumps with mature embryos was similar in ABA concentrations ranging from 3.78 to 15.13 µM in both ‘Linda’ (Fig. 1C) and ‘Sonja’. However, 18.92 µM had negative effect on these cultivars (Table 3). The number of mature embryos in ‘Sonja’ was much lower compared with ‘Linda’. Similar results with ABA application have been obtained by other researchers (NORIEGA and SONDALI 1991; MARCHANT et al. 1996; MURALI et al. 1996). MARCHANT et al. (1996) used 3.8 µM ABA in combination with 2.4-D and GA3 for embryo maturation in R. hybrida ‘Trumpeter’ and ‘Glad tidings’. In addition, Li et al. (2002) obtained both the highest proliferation rate of somatic embryos and proliferation via secondary somatic embryogenesis in R. hybrida ‘Carefree beauty’ with application of 3.8 µM ABA. Exogenous application of ABA has also been used in other species for proliferation and maturation of somatic embryos. OGATA et al. (2005) obtained secondary somatic embryos from carrot seed-coat-derived somatic embryos with application of 1 µM ABA. Mature somatic embryos have been acquired by using 1 µM ABA in Quercus suber (GARCIA-MARTIN et al. 2005) and 18.9 µM ABA in Camellia sinensis (SHARMA et al. 2004). It has been shown that different stresses induce somatic embryogenesis in many plant species (FEHER 2003). It is also known that endogenous ABA increases in many species after various stresses (reviewed by MULBOWROW 2001). It can be concluded that increased levels of endogenous or applied ABA could improve differentiation and maturation of somatic embryos as an adaptation mechanism in response to stress. Molecular and biochemical events underlying this phenomenon are not entirely clear and are yet to be elucidated.

Development of somatic embryos into whole plants

Greening of mature somatic embryos started two weeks after transferring to regeneration media. Germination and subsequent shoot development of somatic embryos occurred within one month of transfer to the regeneration media (Fig. 1D, Table 4). Two different shoot types were observed after two months in these cultures. Morphologically normal shoots developed into proper plant-lets (Fig. 1F) while morphologically abnormal shoots remained undeveloped (Fig. 1E). The highest number of normal shoots (70.0 %) was obtained in medium H containing 1.42 µM NAA, 17.7 µM BAP and 1.4 µM GA3, however the results (60.0 %) were similar to that from medium F which had the same amount of NAA and BAP but lower concentration of GA3 (Table 4).

Different media including various concentrations of PGRs have been used by researchers for germination and shoot regeneration of somatic embryos. BAP, GA3 and an auxin (NAA, IAA or IBA) have been utilized more than other PGRs in the stage of somatic embryo development. ROUT et al. (1991) obtained 12 % normal shoot regeneration from somatic embryos of R. hybrida ‘Landora’ grown on 1/2 MS medium supplemented with 2.2 µM BAP, 0.3 µM GA3 and 24.7 µM adenine sulphate. Likewise, 4.4 µM BAP was used by MARCHANT et al. (1996) for obtaining 17 and 9 % shoot regeneration from mature somatic embryos of R. hybrida ‘Trumpeter’ and ‘Glad Tidings’, respectively. KINTZIস (1999) used 5.2 µM BAP together with 5.7 µM IAA for maturation and germination of somatic embryos in R. hybrida ‘Soraya’ and achieved normal shoot regeneration. VISESSUWAN et al. (1997) applied 8.8 µM BAP for shoot regeneration from somatic embryos of R. hybrida ‘Carl Red’ and R. canina. In the present study, the lowest number of normal shoots were obtained in PGR-free medium (medium P) and also in medium with only NAA (medium A), and their results were significantly different when compared to that from rest of the media containing BAP and GA3. Li et al. (2002) also obtained poor germination of somatic embryos in PGR-free medium. Therefore, it seems that GA3 and BAP are very important for germination and shoot elongation from somatic embryos. GA3 is known for its effect on promoting seed germination by overcoming dormancy in zygotic seeds of somatic species and also for shoot elongation in some species (DAVIES 1995). Since somatic embryos do not have dormancy (DODEMAN et al. 1997; FEHER et al. 2003), GA3 does not seem to be necessary for their germination. However, application of GA3 lowers the endogenous levels of ABA and IAA (DAVIES 1995). Therefore, in the present study, it appears that positive effects of GA3 might be caused by reducing negative effects of ABA which was used for proliferation of somatic embryos. However, no significant differences were observed between 0.29 µM and 1.44 µM of GA3, BAP influences cell division and enlargement, lateral bud growth, leaf expansion and promotion of seed germination in many plant species (MOX et al. 2000). Hence, application of BAP can enhance shoot regeneration and multiplication. Nevertheless, a balance between hormones is required for normal germination and development. It appears that high concentrations of BAP could reduce the germination rate and/or development of somatic embryos. This agrees with the results of MARCHANT et al. (1996) where shoot development was achieved on lower concentrations of BAP compared to the concentrations used for germination of somatic embryos. The results from media B, C, D, E and J with BAP concentrations higher and lower than those in media F and H were not significantly different (Table 4). However, in terms of abnormal shoots, except for medium H which produced the lowest numbers, the results from the rest of the media were not significantly different. A low concentration of an auxin is usually used together with a cytokinin to improve the result.

Secondary somatic embryos were also observed in this stage of development. Different numbers of them produced on developed cotyledons which were mostly abnormal with fused transparent cotyledons. These embryos were not used for further experiments. Bipolar plant-lets were not detected in the present study. Therefore, regenerated shoots were multiplied in MS medium fortified with 0.021 µM NAA and 4.44 µM BAP and adventitious roots were produced on them.

Root production and acclimatization of plants

Close to 100 % of shoots produced adventitious roots on 1/2 MS media containing IAA and IBA. After transferring
to soil, 34 plants (57%) survived and they flowered after 8 to 12 weeks. Regenerated plants were morphologically indistinguishable from the original plants (Fig. 1G–I).

In summary, a protocol for regeneration of miniature rose cultivar via somatic embryogenesis was developed in the present study. This protocol is used currently for genetic transformation work. Moreover, embryogenic callus obtained in the present study has been proliferated and maintained for over three years by sub-culturing every month. Further investigation to achieve higher rate of proliferation, better maturation and to extend life span of somatic embryos would be useful for rose breeding programs.

Acknowledgments

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Paper II

Isolation and characterisation of somatic embryogenesis receptor-like kinase (RhSERK) genes from miniature potted rose (Rosa hybrida cv. Linda)

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Isolation and characterisation of somatic embryogenesis receptor-like kinase (RhSERK) genes from miniature potted rose (Rosa hybrida cv. Linda)

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SUMMARY
The isolation and expression analysis of four partial gene sequences from rose (Rosa hybrida cv. Linda) belonging to the receptor-like kinase gene superfamily is reported. These genes are termed RhSERK1-RhSERK4 (Acc. No. EF631967-70) since they show high identity with genes for the somatic embryogenesis receptor-like kinase (SERK) family in other plant species. A phylogenetic analysis was conducted using SERK proteins from rose and other species. The four isolated RhSERK genes were expressed in both embryogenic callus and mature somatic embryos, indicating a function during the process of somatic embryogenesis in rose. The role of the RhSERK genes is, however, not restricted to somatic embryogenesis, since transcripts for all four genes were found in other tissues as well. All four SERK homologues from rose were expressed differentially in the examined tissues, indicating tissue-specific regulation and suggesting that they have a broader role in plant growth and development.

Somatic embryogenesis is a developmental pathway whereby somatic plant cells undergo embryogenesis in response to different stress conditions or application of plant growth regulators (Feher et al., 2003; Jimenez, 2005). Somatic embryos are useful material for plant propagation, genetic transformation and for molecular and physiological studies on zygotic embryos, and have been reported for more than 200 species to date (Raemakers et al., 1995). Several groups of genes are specifically expressed during somatic embryogenesis, including genes for Somatic Embryogenesis Receptor-like Kinases (SERKs), which belong to the plant receptor kinase superfamily

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(Chugh and Khurana, 2002; Feher et al., 2003; Ito et al., 2005). The plant receptor kinase genes, formally termed receptor-like kinases (RLK), encode proteins, which are characterised by an extracellular domain, a single transmembrane domain and a cytosolic kinase domain (Cock et al., 2002). Several investigations have shown that RLK proteins have a role in controlling a broad range of developmental processes in plants including plant defence responses, brassinosteroid signalling, self incompatibility, nodulation, cell and organ elongation and floral organ abscission (Cock et al., 2002).

The first member of the SERK gene family (DcSERK) was isolated from carrot (Daucus carota) by Schmidt et al., (1997). Since then several SERK genes have been isolated from other plant species such as Dactylis glomerata (DgSERK, Somleva et al., 2000), Arabidopsis thaliana (AtSERK, Hecht et al., 2001), Zea mays (ZmSERK, Baudino et al., 2001), Medicago truncatula (MtSERK, Nolan et al., 2003), Helianthus annuus (HaSERK, Thomas et al., 2004), Theobroma cacao (TcSERK, Santos et al., 2005), Oryza sativa (OsSERK, Ito et al., 2005) and Citrus unshiu (CitSERK1, Shimada et al., 2005). No reports concerning SERK genes in rose (Rosa hybrida) have yet been published.

Since the frequency of somatic embryogenesis is low in some species and the phenomenon is also dependent on the genotype, expression of SERK genes has been used as a marker for recognizing cells, which are competent to develop somatic embryos for some species. DcSERK has been used for this purpose with expression starting in competent single cells and lasting up to the globular developmental stage of the somatic embryos (Schmidt et al., 1997). In Dactylis glomerata, expression of DgSERK followed a similar pattern but it was also expressed at later developmental stages, i.e. in shoot apical meristem, scutellum, coleoptile and coleorhiza (Somleva et al., 2000). In contrast to these species, SERK genes in maize (ZmSERK2) and rice (OsSERK2) were expressed almost uniformly in all investigated embryogenic and non-embryogenic tissues (Baudino et al., 2001; Ito et al., 2005). Thus, before using members of this gene family as a marker for somatic embryogenesis, expression of each SERK gene has to be investigated for the target plant.

As rose, with its globally significant economic importance, is notably recalcitrant with respect to somatic embryogenesis for many cultivars (Burrell et al., 2006; Estabrooks et al., 2007), it is of practical value to investigate SERK gene expression as a potential marker in this species. The aims of the present study were to identify SERK gene homologues in a selected rose cultivar and to investigate the expression of these genes in different tissues.
MATERIAL AND METHODS

Plant material and DNA and RNA extraction

Plants of miniature potted rose (Rosa hybrida cv. Linda) were used as material for the present study. In a previous investigation, a protocol for induction of somatic embryogenesis was developed for this cultivar (unpublished). Non-embryogenic callus was obtained by culturing the in vitro grown leaves of rose on full-MS (Murashige and Skoog, 1962) medium fortified with 90.5 µM 2,4-dichlorophenoxycetic acid and solidified with 3 g l⁻¹ gelrite (Duchefa, The Netherlands). The leaves were wounded transversely on the adaxial surface at 2-4 mm intervals using a scalpel and placed on media with the abaxial side down. Embryogenic callus was obtained after successive sub-culturing of non-embryogenic callus on full-MS medium fortified with 45.4 µM thidiazuron. The embryogenic callus then transferred to half-MS medium containing 7.57 µM abscisic acid for both multiplication and maturation. The embryogenic callus has been sub-cultured and multiplied on this medium for more than two years and is therefore called repetitive embryogenic callus. The matured somatic embryos from this medium are transferred to new media for germination and regeneration. Both embryogenic and multiplication media were solidified with 8 g l⁻¹ agar (Bacto Agar, Becton, Dickinson and Company, USA). All media used contained half- or full-strength MS salts, Staba vitamins (Staba, 1969), 100 mg l⁻¹ myoinositol, 20 g l⁻¹ sucrose and 10 g l⁻¹ glucose.

Genomic DNA from in vitro grown leaves of this cultivar was extracted by means of DNeasy plant mini kit (Qiagen, Hilden, Germany) and used as template for cloning of the SERK genes by PCR. Total RNA was extracted from petals, non-dehiscent stamens, in vitro grown leaves, non-embryogenic callus, repetitive embryogenic callus, matured embryos (cotyledonary stage) and in vitro roots of R. hybrida cv. Linda with RNeasy plant mini kit (Qiagen, Hilden, Germany) and used for RT-PCR expression analysis.

Cloning and sequencing of the RhSERK

The homologous regions among SERK gene sequences from Arabidopsis, carrot, cacao, Citrus and Medicago (accession numbers according to Table II) were identified using the clustal W multiple alignment program (Thompson et al., 1994). Four sets of degenerate primers with degeneracy between four and 96 (Table I) were designed according to these homologous parts and used in all combinations to amplify putative
rose SERK gene sequences. PCR was performed in a Primus 25 advanced (V.08.03, PEQLAB Biotechnologie, GmbH, Germany) thermodcycler and each reaction (with total volume of 25 µl) consisted of 100 ng genomic DNA, 2 U Taq DNA polymerase (BioLabs Inc, NEW ENGLANDS), Taq related reaction buffer (10 mM Tris-HCl, 50 mM KCl and 1.5 mM MgCl₂, pH 8.3), 200 µM dNTPs, primers and sterile water. The amount of each primer set was calculated according to the degeneracy. The PCR conditions were as follows: one cycle of 95°C for 7 min (primary denaturation), 40 cycles of amplification [95°C for 1 min (denaturation), 50°C for 1 min (annealing), 72°C or 1 min (elongation)] followed by a final extension of 72°C for 7 min. PCR products were separated on a 1.5% agarose gel and DNA fragments with expected sizes were purified using E.Z.N.A. gel extraction kit (Omega Bio-Tek Inc., Doraville, GA, USA) and cloned using TOPO TA cloning® Kit (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer and sequenced commercially (MWG-Biotech, Ebersberg, Germany).

TABLE I. Nucleotide sequences of degenerate primers used for cloning of SERK genes from miniature potted rose (Rosa hybrida cv. Linda)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Degeneracy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>5’-TGCAGTTCCAGACCCGAGGT(ACGT)GA(AG)ATGAT-3’</td>
<td>8</td>
</tr>
<tr>
<td>F1</td>
<td>5’- AGATGAT(ACCT)AG(TC)ATGGGC(AGC)GTTC – 3’</td>
<td>18</td>
</tr>
<tr>
<td>F2</td>
<td>5’- TAT CC(ACCT) TA(CT) ATG GC(ACCT) AAT GGA AG – 3’</td>
<td>18</td>
</tr>
<tr>
<td>for</td>
<td>5’- CATGGC(CT)AATGGAAGTGT(CT)G – 3’</td>
<td>4</td>
</tr>
<tr>
<td><strong>Reverse primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5’- CGCCCACCATGGG0(CT)TC(AG)AA(CT)TC -3’</td>
<td>8</td>
</tr>
<tr>
<td>R1</td>
<td>5’- CAT(CT)TA(GT)GG(ATC)GT(CT)TTCCAT(AT)G – 3’</td>
<td>48</td>
</tr>
<tr>
<td>R2</td>
<td>5’- CC(AG) TA(AGC) CCA AA(AG) ACA TC(AG) GT(CT) TTC - 3’</td>
<td>48</td>
</tr>
<tr>
<td>rev</td>
<td>5’- GCATGA(CT)(CT)CC(AG)TA(GCT)CC(AG)AA(AG)AC - 3’</td>
<td>96</td>
</tr>
</tbody>
</table>

Nucleotide sequences obtained from sequencing were compared with available sequences in the databases of the National Centre for Biotechnology Information (NCBI) to find homologous sequences. The tool blastx (http://www.ncbi.nlm.nih.gov/BLAST/) was also used to compare translated sequences to the protein database. Protein sequences encoded by each rose SERK nucleotide sequence were also deduced and compared with each other and with SERK proteins from other species (Table II) using bl2seq (http://www.ncbi.nlm.nih.gov /blast/bl2seq/wblast2.cgi) (Tatusova and Madden, 1999) available on the NCBI home page.
Expression analysis of RhSERKs by RT-PCR

The total RNA extracted from each tissue was treated with RNase-free DNase I (Sigma, Missouri, USA) according to the manufacturer’s instructions to eliminate the remaining genomic DNA in each sample. The DNA-free RNA was used to produce cDNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, CA, USA) according to the protocol of the manufacturer. Two µl of this cDNA were used for each RT-PCR reaction with a total volume of 25 µl. Specific primers for expression analysis of the RhSERK genes were designed according to the sequenced genes. S1-F (5’- TGG CAA AAG AGG AAA CGC -3’) and S1-R (5’- TCC AAT CGT ACC ACG TAC AG -3’) primers were target against RhSERK1, S2-F (5’- GCA GTT CAT CGA AAT CTT CTT C -3’) and S2-R (5’- CAA AGT CTC CAA CAA CAG CC -3’) against RhSERK2, S3-F (5’- TTA GAA CGC CCA CCA CCA CCA C -3’) and S3-R (5’- CAT GGC TTC GAA TTC CTT CTC ATC CAG C -3’) against RhSERK3 and S4-F (5’- AAG TGT TGC TTC GTC GCG TCT C -3’) and S4-R (5’- TGC CAA CCC AAA ATC TCC CAC C -3’) against RhSERK4 to amplify fragments with 213, 292, 168 and 194 bp, respectively. S2 and S4 primers are spanning over an intron and will amplify fragments with 495 and 305 bp for DNA as template, respectively, to show any possible contamination with genomic DNA.

RT-PCR was carried out using the thermocycler and the basic PCR program as described for cloning of SERK genes above. However, annealing temperature and the number of cycles were different for each pair of SERK primers. Except for S3, where 54°C was used for annealing, 50°C was applied as annealing temperature for the rest of the SERK primers. Amplification was carried out with 30, 33, 35 and 37 cycles for S1, S2, S3 and S4 primer pairs, respectively.

As control for the expression studies with RT-PCR, rose β-actin was cloned. First, β-actin encoding gene from Solanum tuberosum (Accession number: X55751) was used to design primers for cloning of rose actin, and then PCR was conducted with these primers on rose genomic DNA. The 308 bp fragment was cloned and sequenced as described for cloning of SERK gene in previous section. Specific primers for RhActin, Act-F (5’- TTG CTG GAC GAG ATT TAA C AG AC -3’) and Act-R (5’- GAA ATA GGA CTT CAG GGC AAC G -3’), were designed from the obtained sequence and used to amplify a 249 bp fragment which was used as reference in the expression analysis.
For expression analysis of *RhSERK1*, *RhSERK2*, *RhSERK3* and *RhSERK*, each PCR-reaction was repeated three times and the products were separated by electrophoresis in 1% agarose gel and visualised by Ethidium bromide (EtBr) under UV light. The signal intensities were quantified with Quantity One Software (Bio-Rad, CA, USA). The intensity of each signal was corrected by division with the density of the EtBr stained actin band, and given as % of the strongest signal on the blot.

**Phylogenetic tree**

Sequence alignments were made using Clustal W software (Thompson *et al.*, 1994) with SERK protein sequences from *Arabidopsis* (AtSERK1-5), *Citrus unshiu* (CitSERK1), carrot (DcSERK), cacao (TcSERK), *Medicago truncatula* (MtSERK) and deduced amino acid sequences of rose SERKs (RhSERK1-4). The phylogenetic tree was made by the MegAlign (DNAStar, Ver. 5.01) program using the aligned amino acid sequences to view the evolutionary relationships predicted from the multiple sequence alignment. The length of each pair of branches in this tree represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

**RESULTS**

**Cloning and sequencing of the RhSERK and RhActin**

Fragments with the expected sizes were amplified from the primer sets F1-R2, F2-R and F2-R2 and sequence comparison with NCBI GenBank sequences revealed high identity with SERK sequences from other species (Table II). Although other sequences also showed some homology to these rose sequences the highest homology was to SERK genes. These partial sequences, with 660, 595, 381 and 404 bp, respectively, were called *RhSERK1-4* and submitted to NCBI GenBank (accession numbers EF631967-70). Alignments of these sequences with SERK sequences from other species revealed that all of them contain an intron. These partial sequences are encoding parts of the kinase domain in related SERK proteins (Figure 1A). Deduced amino acid sequences of these partial SERK genes, especially *RhSERK1* and *RhSERK2*, have high identity with SERK homologues from AtSERK1-5, CitSERK1, DcSERK, TcSERK and MtSERK1 (Table II). *RhSERK2* has very high identity in amino acid sequence with other SERK proteins showing 100% identity with AtSERK1 (Figure 1B) and TcSERK, 99% identity with CitSERK1 and 98% identity with MtSERK1. The nucleotide sequence of *RhSERK1* is 85, 86 and 75% identical to that of *RhSERK2*, *RhSERK3* and *RhSERK4*, respectively.
RhSERK2 is 97 and 77% identical to RhSERK3 and RhSERK4 and finally RhSERK3 has no identity to RhSERK4. Identity among the four rose SERKs at the amino acid level is between 73 and 98% (Table II).

TABLE II. Percent protein identity among partially sequenced Rosa hybrida SERKs (RhSERK1-RhSERK4) and SERKs from Arabidopsis (AtSERK1-5), Citrus unshu (CitSERK1), carrot (DcSERK), cacao (TcSERK) and Medicago (MtSERK). (Accession numbers of Arabidopsis are from TAIR and for the other species from NCBI GenBank)

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Acc. No</th>
<th>RhSERK1</th>
<th>RhSERK2</th>
<th>RhSERK3</th>
<th>RhSERK4</th>
</tr>
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<tr>
<td>AtSERK1</td>
<td>2013020</td>
<td>94</td>
<td>100</td>
<td>98</td>
<td>76</td>
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<td>94</td>
<td>88</td>
<td>70</td>
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<tr>
<td>AtSERK3</td>
<td>2119259</td>
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<td>96</td>
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<td>74</td>
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<tr>
<td>AtSERK4</td>
<td>2040460</td>
<td>89</td>
<td>91</td>
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</tr>
<tr>
<td>AtSERK5</td>
<td>2040470</td>
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<td>90</td>
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<td>72</td>
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<tr>
<td>CitSERK1</td>
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<td>99</td>
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<td>75</td>
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<tr>
<td>DcSERK</td>
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<td>96</td>
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<tr>
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<td>100</td>
<td>98</td>
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<td>-</td>
<td>-</td>
<td>75</td>
</tr>
<tr>
<td>RhSERK4</td>
<td>EF631970</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>

The partial actin sequence amplified from rose with 264 bp showed 75% identity in nucleotide sequence and 87% in deduced amino acid sequence with that of β-actin from potato (Acc. No. X55751). This sequence does not contain an intron and has been submitted to the NCBI GenBank as RhActin (accession number EF631971).

Expression analysis of RhSERKs by RT-PCR

Expression analysis using specific primers targeted against RhSERK1-4 showed that these genes are expressed with different levels in all examined tissues from petals, stamens, leaves, non-embryogenic callus, repetitive embryogenic callus, matured embryos and roots (Figure 2). The four investigated rose SERK genes all appear to be regulated in a tissue-specific manner, since none of them had identical expression in all investigated tissues. The expression patterns of RhSERK1, RhSERK2 and RhSERK4
exhibited greater similarity to each other than to \textit{RhSERK3} expression. Transcript levels of this group of three SERK genes were higher in stamens and roots and lower in petals when compared to \textit{RhSERK3}.

\textit{AtSERK1} (3258 bp)

\begin{center}
\begin{tabular}{c|c|c|c}
 & Extracellular domain & Transmembrane domain & Kinase domain \\
\hline
\textit{RhSERK1} (660 bp) & 18 bp & 355 bp & 287 bp \\
\textit{RhSERK2} (595 bp) & 108 bp & 203 bp & 284 bp \\
\textit{RhSERK3} (381 bp) & 20 bp & 198 bp & 163 bp \\
\textit{RhSERK4} (404 bp) & 21 bp & 111 bp & 272 bp \\
\end{tabular}
\end{center}

FIG. 1. Schematic comparison of the partially sequenced SERK genes from rose (\textit{RhSERK1-4}) with a \textit{SERK} gene from \textit{Arabidopsis} (\textit{AtSERK1}). White boxes are exons and grey boxes are introns (not to scale). (A). Alignment of \textit{RhSERK1-4} and \textit{AtSERK1} with Clustal W. Amino acids marked with asterisks (*) indicates conserved regions. The dark arrow between R and E amino acids shows the position of introns (B).
Phylogenetic tree

The phylogenetic analysis summarizes the phylogenetic relationships, as deduced from the partial protein sequences of the four RhSERK proteins to each other and other SERK proteins (Figure 3). The RhSERK4 sequence is relatively distantly related to RhSERK1-3 and the other SERK sequences.

FIG. 2. Expression levels of RhSERK genes obtained by RT-PCR. RNA was extracted from different rose tissues: (P) petals; (S) stamens; (L) leaves; (NEC) non-embryogenic callus; (EC) embryogenic callus; (ME) matured embryos, and (R) roots. The intensity of each signal was corrected by division with the density of the EtBr stained Actin band, and given as % of the strongest signal on the blot.

DISCUSSION

Different numbers of SERK genes have been isolated from different plant species with the highest number (five) from Arabidopsis (Hecht et al., 2001). Maize and sunflower both have four homologues (Baudino et al., 2001; Thomas et al., 2004) and one copy of SERK has been isolated from carrot (Schmidt et al., 1997), cacao (Santos et al., 2005), Citrus (Shimada et al., 2005) and Medicago truncatula (Nolan et al., 2003). In
the present study four homologues of SERK genes, designated *RhSERK1*-*RhSERK4*, have been isolated from rose (*Rosa hybrida* cv. Linda). *RhSERK2* and *RhSERK3* show high (98%) identity indicating that the genes might be a result of a duplication event.

Expression analysis of *RhSERK1*-4 showed that all genes were expressed differentially in the investigated tissues (Figure 2). The phylogenetic analysis showed that, at the amino acid level, *RhSERK4* show the lowest identity (73-76%) to the other *RhSERK*ks. Despite this difference, the expression pattern of *RhSERK4* is more similar with that of *RhSERK1* and *RhSERK2* than with *RhSERK3*. Although *RhSERK2* and *RhSERK3* showed high identity in both nucleotide and amino acid sequence, their expression patterns were different (Figure 2). The expression pattern for *RhSERK3* is different from the other rose SERK genes, being higher in petals and lower in stamens and roots (Figure 2). This might indicate a somewhat different function for this gene.

FIG. 3. Phylogenetic tree of SERK family protein kinases based on deduced amino acid sequences for *RhSERK*ks and available protein sequences of other SERKs in GenBank. The sequences were aligned by the Clustal program using PAM 250 residue weight table. The scale indicates sequence pair distances. The length of each pair of branches represents the evolutionary distance between sequence pairs. This is measured with the scale below the tree indicating the % amino acid substitutions (with corrections for the possibility of more than one substitution) between the protein sequences. Names of the SERK protein homologues are as in Table II.
Expression analysis showed that all RhSERK genes are expressed in both embryogenic callus and mature somatic embryos, indicating that these genes may be active during the process of somatic embryogenesis in rose. In other studies expression of DcSERK genes in carrot was observed in embryogenic callus and lasted up to the globular stage of somatic embryos (Schmidt et al., 1997) whereas in Dactylis glomerata expression of DgSERK ended at the globular stage but was observed in later embryo stages in both shoot and radical meristems (Somleva et al., 2000). In Arabidopsis thaliana, expression of AtSERK1 lasted until the heart stage of somatic embryos, with a weak signal being detected in adult vascular tissues (Hecht et al., 2001).

The expression of all of the rose SERK genes in the wide range of tissues examined in this study, including both embryogenic and non-embryogenic ones, reinforces the suggestion that there is a wider role for these genes in plant development. Results reported in rice (Ito et al., 2005), maize (Baudino et al., 2001) and Citrus unshiu (Shimada et al., 2005) support this assertion. Expression of OsSERK2 in rice was uniform in all examined tissues but expression of OsSERK1 showed varying levels in the different samples tested (Ito et al., 2005). Similarly, expression of ZmSERK2 in maize was relatively uniform in all of the embryogenic and non-embryogenic samples, whereas ZmSERK1 expression was markedly variable in the different tissues investigated and was not expressed in mature leaves and root tips (Baudino et al., 2001). In Citrus unshiu the expression of CitSERK1 was also detected in various embryogenic and non-embryogenic tissues but not in proliferating, non-embryogenic calli or in fruits 180 days after flowering (Shimada et al., 2005).

However, as with OsSERK1 and ZmSERK1, rose SERK genes are expressed differentially in the investigated tissues, which may indicate a level of tissue specificity in these genes. Similarly, the SERK gene from cacao (TcSERK) was expressed in initial induced and repetitive embryogenic callus, leaves, somatic and zygotic embryos but was not expressed in staminoids, petals and roots, which suggests a particular role for this gene in the development of cacao embryos (Santos et al. 2005).

In conclusion, the expression patterns of the four investigated rose SERK gene homologues show that the function of these genes are not restricted to somatic embryogenesis and may play a wider role in plant growth and development. Consequently, they would be of little use as a marker to distinguish embryogenic competent cells. Isolation of the SERK promoters and transformation with GUS (β-glucoronidase) constructs and subsequent spatial expression analysis might be a helpful
approach for future studies aiming at a better understanding of the role of these genes in rose.

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Agrobacterium - mediated transformation of miniature potted rose \textit{(Rosa hybrida cv. Linda)} with $P_{SAG12}$-IPT gene for retardation of leaf senescence

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(In preparation)
Agrobacterium - mediated transformation of miniature potted rose (Rosa hybrida cv. Linda) with P_{SAG12}-IPT gene for retardation of leaf senescence

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Abstract

Transgenic plants of Rosa hybrida ‘Linda’ were obtained via transformation with Agrobacterium tumefaciens strain harboring the binary vector pSG529(+) containing the P_{SAG12}-IPT construct. Embryogenic callus was used as explant for transformation experiments. Among the A. tumefaciens strains AGL1, GV3850 and LBA4404 (containing P_{35S}-INTGUS gene) used in transformation, transgenic shoots were obtained only when AGL1 was used. The highest transformation frequency was 10% and it was achieved when ½ MS medium was used for the dilution of overnight culture of Agrobacterium. Transformation frequency was lower (3.3%) when YEP medium used for dilution. No results were obtained when LB or Min-A media were used for dilution. Southern analysis confirmed integration of 1-6 copies of the NPTII gene into the rose genome. Four transgenic lines were obtained which were morphologically true-to-type and indistinguishable from control shoots while they were in in vitro cultures. However, some abnormalities were observed in several greenhouse grown plants established from one of the transgenic lines. Adventitious root induction was more difficult in transgenic shoots compared to the control shoots, however, one of the transgenic lines was rooted and the plantlets were transferred to the greenhouse for phenotypical analysis. (Final analyses have not been done at the time of thesis submission, however, preliminary experiments on in vitro shoots showed that transgenic plants stayed green for a longer period than the control plants after exposing to dark stress. More analyses on the greenhouse-grown plants are required to confirm the results.)

Key words: Leaf senescence, Cytokinin, ipt, stress, postharvest, rose breeding

Abbreviations: IPT, isopentenyl transferase; SAG, senescence-associated gene
Introduction

Leaf senescence is the sequence of biochemical and physiological events in the final stage of development from mature, fully expanded state until death (Smart 1994). Despite of its detrimental effects in agricultural products, it is important in nutrient relocation of the leaf and therefore it is a critical process for the fitness of plants and regarded as an evolutionarily acquired genetic process (Gan and Amasino 1997; Nam 1997; Smart 1994). However, several studies have been carried out for better understanding of this process and reducing its detrimental effects in different crops including ornamental plants.

Several experiments have shown that cytokinins are the most important senescence-retarding growth regulators (Buchanan-Wollaston 1997; Gan and Amasino 1996; Lim et al. 2007). It has been demonstrated that senescence is accompanied by a decline in leaf content of cytokinin, and it has also been shown that spraying with artificial or natural cytokinin prevents leaf yellowing in some plants (Gan and Amasino 1996; Weaver et al. 1998). In miniature potted rose, spraying with cytokinins has reduced leaf yellowing (Clark et al. 1991; Tjosvold et al. 1994) and increased flower longevity (Serek and Andersen 1993).

The enzyme which catalyses the first and rate limiting step of cytokinin biosynthesis pathway is called isopentenyl transferase (IPT) and has been isolated from *Agrobacterium tumefaciens* (Akiyoshi et al. 1984) and *Arabidopsis thaliana* (Takei et al. 2001). A gene encoding this enzyme is called *ipt* and has been well characterized in *A. tumefaciens* (Akiyoshi et al. 1984; Kakimoto 2003) and the effectiveness of the *ipt* gene in stimulating cytokinin biosynthesis has been demonstrated in several plant species under control of different promoters (summarized by Gan and Amasino 1996). Since the uncontrolled expression of *ipt* gene after induction of the promoter leads to the overproduction of cytokinin which causes a range of effects including physiological and morphological abnormalities, an autoregulatory senescence-inhibition system was established using a suitable promoter preventing cytokinin overproduction (Gan and Amasino 1995 and 1996). In this system, the promoter region of *SAG12*, a highly senescence-specific gene (Lohman et al. 1994), from *Arabidopsis* is fused to the *ipt* gene from *A. tumefaciens* and the chimeric gene (PSAG12-IPT) which is called gene in the present study, was used for transformation of tobacco plants (Gan and Amasino 1995). Transgenic plants showed a significant delay in leaf senescence, but no further abnormalities were noted in plant growth and development compared to wild-type plants.
The reason is that the increased levels of cytokinin reduce leaf senescence and attenuate the expression of the *ipt* gene and consequently prevent cytokinin overproduction (Gan and Amasino 1995). This gene has been transferred successfully to several plant species including tobacco (Gan and Amasino 1995; Jordi et al. 2000), jasmine tobacco (Schroeder et al. 2001), broccoli (Chen et al. 2001), lettuce (McCabe et al. 2001) and *Petunia* (Chang et al. 2003; Clark et al. 2004). In the present study, we have successfully transformed a miniature potted rose cultivar with this gene.

Successful transformation of rose has been achieved via biolistic methods (Marchant et al. 1998a, b), *Agrobacterium rhizogenes* (Firoozabady et al. 1994) and *A. tumefaciens* (Condliffe et al. 2003; Derks et al. 1995; Dohm et al. 2001; Firoozabady et al. 1994; Li et al. 2002; Van der Salm et al. 1997). The type of explant is an important factor and it must be suitable for regeneration allowing the recovery of whole transgenic plants (De la Riva et al. 1998; Slater et al. 2003). Different explants including stem slices (Van der Salm et al. 1997), embryogenic callus (Condliffe et al. 2003; Derks et al. 1995; Firoozabady et al. 1994; Kim et al. 2004; Li et al. 2002) and somatic embryos (Dohm et al. 2001) have been used for successful transformation of different rose cultivars. The choice of *Agrobacterium* strain is also a major factor contributing to the success or failure of the transformation especially for crops which are difficult to transform (Slater et al. 2003). Different strains of *Agrobacterium* have been used for transformation of rose cultivars. Another factor which affects transformation efficiency is the media used for dilution of *Agrobacterium* culture before inoculation. Different media have been used for this purpose in transformation of rose cultivars including YEP (Dohm et al. 2001), MS (Derks et al. 1995; Li et al. 2002) and Min-A (Dohm et al. 2001; Firoozabady et al. 1994) media.

Available reports on rose transformations are mostly dealing with garden roses or cut roses. To our knowledge, no successful transformation of miniature potted rose has been reported. In the present study, we report the transformation of miniature potted rose cultivar, *R. hybrida* ‘Linda’ using different *Agrobacterium* strains and different inoculation media, in order to reduce leaf senescence.

**Materials and Methods**

**Plant material**

Embryogenic calli containing mature somatic embryos of miniature potted rose (*Rosa hybrida* cv. Linda) were used as explants for transformation in the present study. In a previous investigation, a protocol for induction of somatic embryogenesis was developed
for this cultivar (Zakizadeh et al. 2008). Non-embryogenic callus was obtained by culturing the in vitro grown leaves of rose on full-MS (Murashige and Skoog 1962) medium fortified with 90.5 µM 2,4-dichlorophenoxyacetic acid and solidified with 3 g l⁻¹ gelrite (Duchefa, The Netherlands). Embryogenic callus was obtained after successive sub-culturing of non-embryogenic callus on full-MS medium fortified with 45.4 µM thidiazuron. The embryogenic callus has been subcultured and multiplied on half-MS medium fortified with 7.57 µM abscisic acid (ABA) for more than two years and was used for transformation with several Agrobacterium strains. Media for embryogenesis and multiplication were solidified with 8 g l⁻¹ agar (Bacto Agar, Becton, Dickinson and Company, USA). All media were based on half- or full-strength MS salts, Staba vitamins (Staba 1969), 100 mg l⁻¹ myoinositol, 20 g l⁻¹ sucrose and 10 g l⁻¹ glucose.

**Agrobacterium** strains and plasmids

The binary vector pSG529(+) harboring the cytokinin biosynthesis gene, isopentenyl transferase (0.7 kb), originated from A. tumefaciens and binary vector pSG514 harboring the *uidA* gene (1.86 kb) coding for β–glucuronidase (GUS), both under the control of senescence-associated gene (SAG) promoter SAG12 (2.2 kb) from Arabidopsis (Gan and Amasino 1995) were used for this study. Both vectors were carrying nptII (neomycin phosphotransferase II) gene under the control of CAMV 35 S promoter and they were gifts from Prof. R. M. Amasino (University of Wisconsin, Madison, WI) and Prof. Susheng Gan (Cornell University). These plasmids were multiplied in *E. coli* cells and introduced into GV3850 and AGL1 strains of *A. tumefaciens* by electroporation.

*A. tumefaciens* strain LBA4404 harboring plasmid pBI121 containing the genes nptII and *uidA* (accession no. AF485783), under the control of the NOS and the CaMV35S promoters, respectively, was also used as a control for transformation.

**Agrobacterium-mediated transformation**

Bacterial strains were cultured in liquid LB medium (Sambrook and Russell 2001) supplemented with 50 mg l⁻¹ rifampicilin and 20 mg l⁻¹ gentamicin for overnight at 28 °C on a rotary shaker with 220 rpm. The resulting *Agrobacterium* suspensions were diluted at 1:20 ratio in different media including LB, YEP (Sambrook and Russell 2001), Min-A (Ausubel et al. 2002) or half-MS, all supplemented with 200 µM acetosyringone and shaken under the same condition to reach OD₆₀₀ about 0.8-1.0. Ten clumps of embryogenic callus, each one weighed about 100 mg, were used for transformation with each bacterial suspension. Inoculation with *Agrobacterium* was carried out for one h at
28°C on a rotary shaker at 100 rpm. Explants then were blotted on filter paper and placed on Petri dishes containing half-MS salts fortified with 7.57 µM ABA and 200 µM acetosyringone and cocultivated in the dark for three days. The embryogenic callus was then washed three times with sterile deionized water containing 500 mg l⁻¹ cefotaxime, 150 mg l⁻¹ Timentin (mixture of ticarcillin disodium and potassium clavulanate in 15:1 ratio) and 100 mg l⁻¹ vancomycin and then blotted and transferred to the same medium but without acetosyringone and with the same concentration of antibiotics which was used for the washing stage, to inhibit further bacterial growth.

Selection of transformants and plant recovery

After two weeks, embryogenic callus was transferred to the same medium containing 60 mg l⁻¹ kanamycin for selection. Embryogenic callus was subcultured once every month. All cultures were kept at 25±2 °C in a 16 h photoperiod. Each treatment was repeated three times and each replication consisted of one Petri dish containing 10 explants.

Kanamycin resistant embryogenic callus was separated from darkened or bleached tissues during each subculture. Germination and subsequent shoot development of matured putative transgenic somatic embryos were carried out as described previously (Zakizadeh et al. 2008). In brief, they were transferred to the medium containing 1.42 µM 1-naphthaleneacetic acid (NAA), 17.76 µM 6-benzylaminopurine (BAP) and 1.44 µM gibberellic acid (GA₃) but without kanamycin. Regenerated transformed shoots were separated from the rest of the explants and were transferred to the shoot multiplication medium containing MS salts fortified with 0.021 µM NAA and 4.44 µM BAP in addition of 100 mg l⁻¹ kanamycin. For adventitious root induction, developed shoots of one transgenic line were transferred to MS medium containing half-strength salts and half sucrose fortified with 17.1 µM IAA and 2.46 µM IBA. Rooted plantlets were then transferred to 9 cm plastic pots containing peat (Pindstrup Mosebrug A/S Denmark) and covered with transparent polyethylene bags in the greenhouse. Plants were watered with 1 gl⁻¹ Octave (BAFS, AGRO BV, Arnhem, The Netherlands) and 3 ml l⁻¹ Bactimos (Abbott, North Chicago) to protect them from fungal and larvae attack, respectively. Plants were maintained under greenhouse conditions at 19 ± 1 °C and 16 h photoperiod (provided by SON-T high pressure sodium lamps with 600 µmol m⁻² s⁻¹ at plant surface). The cover was removed gradually within two weeks and the established plants were grown until flowering and used for phenotypical analysis.
Confirmation of transformation with PCR

After pre-screening on kanamycin containing media, seven putative transgenic lines were selected and genomic DNA from *in vitro* grown leaves of these lines was extracted with DNeasy plant mini kit (Qiagen, Hilden, Germany) and was used as template for PCR to confirm transformation. For detection of the *nptII* gene, a set of primers including forward 5'-GAGGCTATTCGGCTATGACTG-3' and reverse 5'-ATCGGGAGCGGCGATACCGTA-3’ primers were used to amplify a 0.7 kb fragment of the *nptII* gene. For detection of a fragment containing a segment of the SAG12 promoter and the *ipt* gene, forward primer 5’-ATCTCTATAGTACACAAGTAGAGA-3’ and reverse primer 5’-GAGCGATCCCATGAATCAAC-3’ were used to amplify a 996 bp fragment. PCR was performed in a Primus 25 advanced (V.08.03, PEQLAB Biotechnologie, GmbH, Germany) thermocycler and each reaction (with total volume of 25 µl) consisted of 200 ng genomic DNA, 2 U Taq DNA polymerase (BioLabs Inc, New England), *Taq* related reaction buffer (10 mM Tris-HCl, 50 mM KCl and 1.5 mM MgCl₂, pH 8.3), 200 µM dNTPs, primers and sterile water. The PCR conditions were as follows: one cycle of 95°C for 7 min (primary denaturation), 40 cycles of amplification [95°C for 1 min (denaturation), 52°C for 1 min (annealing), 72°C or 1 min (elongation)] followed by a final extension of 72°C for 7 min. PCR products were separated on a 1% agarose gel and visualised by Ethidium bromide (EtBr) under UV light. Sizes of the amplified fragments were determined by comparison to a 100 bp DNA ladder (BioLabs Inc, New England).

Southern blot analysis

To confirm that the chosen lines were independent transgenic lines, and to determine copy number of the transgene in the obtained lines, Southern blot analysis was carried out. Genomic DNA was extracted from seven transgenic lines and control plant using CTAB method (Keb-llanes et al. 2002). About 10 µg of isolated genomic DNA from each transgenic line and control plant was digested overnight with 30 units of either *Bam*HI or *Hind*III restriction enzymes (Fermentas) at 37°C and subsequently electrophoresed in a 0.8% (w/v) agarose gel in TAE buffer at 30 V for about 18 h. The DNA was depurinated in 0.25 M HCl and denatured in 0.5 M NaOH, 1.5 M NaCl. Following neutralization, the DNA was transferred to Hybond-N⁺ nylon membranes (Amersham) in 20X SSC (3 M NaCl, 0.3 M Na-citrate, pH 7.0). After cross-linking by irradiation with UV light, the membranes were rinsed with water and stored at 4°C until hybridization. The 515 bp nptII probe was labeled with DIG (digoxigenin) (Roche) by PCR using plasmid pBEO210 (accession number EF192606) and the primers (forward primer 5’-TGAATGAACTGCAGGAGA-3’ and reverse primer 5’-AATATCAGCGG
GTAGCCAAACG-3'). The DIG-labeled probe was denatured at 95°C for 10 min and stored on ice until use. The membranes were pre-hybridized in 15 ml of Dig Easy Hyb (Roche) at 42°C for 30 min. This was followed by hybridization overnight at 42°C with 100 ng of denatured DIG-labeled probe in 7 ml of Dig Easy Hyb (Roche). Post-hybridization washes and DIG-detection were performed according to the supplier (Roche). X-ray films (Amersham) were exposed to the membranes for about 1 h. Fragment sizes were estimated by comparison with DNA MW Marker III (0.56–21.2 kb, Roche, 1 lg).

Senescence retardation analysis

As rooted control plants are not ready for the experiment, this part of the study has not been started yet. However the procedure will be like this: two experiments will be carried out using both transgenic and control greenhouse grown plants. Plants will be exposed to either dark or drought stress for a certain period of time according to the preliminary experiments. During the stress period changes in phenotype of plants will be monitored. For molecular analysis, total RNA will be extracted from old and young leaves of both transgenic and control plants. Leaves will be harvested before applying the stress and at selected time points after stress treatment. Total RNA will be used for producing cDNA for further RT-PCR expression analysis. Chlorophyll content of the leaves will be extracted from transgenic and wild-type plants before and after stress in both experiments, and measured using a spectrophotometer. Each treatment will be repeated three times and each replication will be single potted plant. However, since changes in phenotypes of commercially grown plants can reduce their marketable values, recovery of true to type transgenic plants is critical in rose breeding (Dohm et al. 2001). Therefore, besides retardation in leaf senescence, phenotypes of transgenic lines will be monitored carefully during stress treatment and will be compared with control plants. Phenotypic comparisons will comprise traits as leaf shape, number of leaflets per leaf, length of internodes, stem diameter and flower shape and color.

Statistical analysis

All data will be analyzed using SAS statistical analysis package (Version 8.02). Statistical significances among mean values will be assessed using Duncan’s multiple range tests at $P < 0.05$. 
Results

Plant transformation, selection and regeneration of transformants

*Agrobacterium* strains GV3850 and AGL1 both harboring binary vector pSG529(+) containing \( \text{P}_{\text{SAG12}} \)-IPT or binary vector pSG514 containing \( \text{P}_{\text{SAG12}} \)-GUS were used in preliminary experiments, but due to lack of explants, strains containing \( \text{P}_{\text{SAG12}} \)-GUS were not used any further. Embryogenic callus were subcultured for about five months on kanamycin containing media before matured cotyledonary embryos can be observed. Primary shoots regenerated from these putative transgenic embryos could not survive on kanamycin containing media. Therefore, antibiotic free media were used in this stage. Final results showed that transgenic shoots obtained only from inoculation with AGL1 strain. No result was obtained from treatments with GV3850 and LBA4404. Frequency of transformation was 10% when *Agrobacterium* solution was diluted in half-MS medium while it was only 3.3% when YEP medium was used for dilution. No transgenic shoot was obtained when *Agrobacterium* solution was diluted in LB or Min-A media. Overgrowth of explants with *Agrobacterium* was observed more when LBA4404 strain was used. Also when YEP or LB media were used for dilution of *Agrobacterium* and inoculation of explants. Cefotaxime and Timentin which were used in preliminary experiments were not enough for preventing the overgrowth with *Agrobacterium* and therefore, vancomycin was added to the washing solution and to the media. Regenerated shoots were propagated on shoot multiplication medium and were screened on kanamycin containing medium. Shoots which survived on 100 mg l\(^{-1}\) kanamycin were supposed to be transgenic and were used for DNA extraction. Non-transformed shoots turned brown and died on kanamycin supplemented media. Moreover, *in vitro* grown shoots of transgenic plants were shorter and leaves were thicker compared to those of control plants.

Adventitious root induction

A large amount of undifferentiated callus was produced at the basipetal end of transgenic shoots on rooting media. Root induction was very difficult in these shoots but, adventitious roots finally formed on some of transgenic shoots after using higher concentrations of IAA and lower concentration of IBA. This was in contrast with results of root induction on control shoots where lower concentration of IAA and higher concentration of IBA were used. Root induction on transformed shoots took almost two months compared with two weeks on control plants. Rooted plantlets were transferred to the greenhouse and after acclimatization they were grown until flowering.
Confirmation of transformation

After selection of transgenic shoots on kanamycin containing media, DNA extracted from these shoots was used for analysis of transformation. Only shoots which survived on 100 mg l\(^{-1}\) kanamycin were used for further analysis. Confirmation with PCR showed that pre-screening of transformants on kanamycin was 100% efficient. All selected lines were transgenic and contained both SAG12-IPT and NPTII fragments (Fig. 1).

Figure 1. Confirmation of transformation of selected putative transgenic lines using PCR. Identification of fragments with expected sizes resulting from SAG12 and IPT primers (left) and nptII primers (right) confirming the presence of the transgene in all selected lines. No product has been amplified using these primers in control plants (lane 8). Lanes: P, plasmid; M, 100 bp marker; 1-7, transformants; 8, control.

Southern analysis using \textit{nptII} probe confirmed transformation of all selected lines and revealed the integration of 1-6 copies of the \textit{nptII} gene into the rose genome (Fig. 2). Even though all seven primarily selected lines were transformed, Southern analysis demonstrated that four of these lines were different. Lines 1 and 2 with six copies of the inserted gene were identical and lines 3, 4 and 5 with two copies of the inserted gene were identical. Lines 6 and 7 with one and two (or six) copies, respectively, are independent resulting from different transformation events (Fig. 2). However, there is a discrepancy between the results of the two enzymes with line 7 and therefore the blot will be redone.

Senescence retardation analysis

\textit{Since the main experiment has not started yet, a small preliminary experiment was carried out with in vitro grown shoots to study the transgenic plants during senescence.} Shoots from both transgenic and control plants were exposed to dark stress for about
three weeks and were observed every few day for phenotypical changes. The results showed that transgenic plants stayed green for longer period than control ones. Greenhouse experiments are needed to validate or confirm these results. The results of this preliminary experiment including figures are discussed in the final discussion of the thesis.

Figure 2. Southern blot analysis of transgenic plant lines. Genomic DNAs from all selected lines were digested with either BamHI (left) or Hind III (right) and probed with a labeled 515 bp nptII amplicon. Results show the existence of four transgenic lines among seven primarily selected lines. Lines 1 and 2 with six copies of the inserted gene were identical and lines 3, 4 and 5 with two copies of the inserted gene were identical. Lines 6 and 7 with one and two (or six) copies, respectively, are independent resulting from different transformation events. Lanes: M, 0.56–21.2 kb molecular maker III; 1-7, transformants; 8, control

**Discussion**

Since the first transformation of cut rose, *R. hybrida* ‘Royalty’, 14 years ago (Firoozabady et al. 1994), less than 10 successful transformation studies have been reported in different rose cultivars using *Agrobacterium* strains (Condliffe et al. 2003; Derks et al. 1995; Dohm et al. 2001; Kim et al. 2004; Li et al. 2002; Van der Salm et al. 1997). This indicates the difficulties in transformation of this species due to the recalcitrant nature of this genus like many other woody species. Most of the available protocols are also genotype dependent. Moreover, most of studies have been carried out in either cut rose or garden roses and no transformation studies of miniature-potted rose have been reported so far. It seems necessary to establish an efficient regeneration/
transformation protocol for each cultivar of interest. In the present study, transgenic plants of miniature-potted rose, *R. hybrida* ‘Linda’ were obtained via *Agrobacterium*-mediated transformation. The highest transformation frequency was 10% in this study. Transformation frequency in rose has been reported to be very low. Although high transformation frequency has been reported in callus (Li et al. 2002) or somatic embryo (Derks et al. 1995) stages, further development to generate transgenic plants was not reported. Embryogenic callus was used as explants in this study, and similar studies have used such embryonic tissue in other roses (Condliffe et al. 2003; Derks et al. 1995; Firoozabady et al. 1994; Kim et al. 2004; Li et al. 2002).

Among the *Agrobacterium* strains which were used in the present study, only AGL1 strain gave successful results. For recalcitrant plant species, the choice of strain is a major factor contributing to the success or failure of the transformation (Slater et al. 2003). Different *Agrobacterium* strains such as LBA4404, AGL0, EHA101, EHA105, C58C1, GV3101 and GV2260 have been used previously for transformation of rose cultivars. The two strains, AGL1 and GV3850, were used for the first time in rose transformation, and the results showed that AGL1 is more efficient in transforming the rose cultivar studied. However, since several other factors have been shown to influence transformation frequency, it can not be concluded that GV3850 is not suitable for transformation of rose cultivars.

The media used for dilution of *Agrobacterium* suspension before inoculation can affect the transformation frequency. Different media have been used for this purpose in rose transformation including YEP (Dohm et al. 2001), MS (Derks et al. 1995; Li et al. 2002) and Min-A (Dohm et al. 2001; Firoozabady et al. 1994). In the present study, dilution in ½ MS medium resulted in higher transformation frequency (10%) compared to the dilutions in YEP (3%), LB (0%) or Min-A (0%). These results are in contrast to the results of Dohm et al. (2001) where dilution of the *Agrobacterium* culture in Min-A medium resulted in higher transformation rates than dilution in YEP medium. This difference might indicate the effects of different factors including genotype, *Agrobacterium* strains, cocultivation period and antibiotics on transformation frequency. Moreover, use of acetosyringone in the present study might be one of the reasons for this difference. Artificial wounding or adding acetosyringone is usually done during the transformation procedure. However, these treatments are not compulsory when embryogenic callus or somatic embryos are used as explants (Dohm 2003).
An advantage of *Agrobacterium*-mediated transformation over biolistic methods is claimed to be transformants with lower copy number of the transgene, but transgenic lines with up to 6 copies of transgene were obtained in the present study. Southern blot analysis revealed the existence of four transgenic lines with 1, 2 and 6 copies of the integrated gene (Fig. 2). The transgene copy number per integration site has been associated with gene silencing and this could create problem in developing desirable transgenic plants (Li et al. 2002). However, gene silencing has not been described for transgenic roses (Dohm 2003). The perfect transformation contains a single copy of the transgene which will segregate as mendelian trait. Variability observed among transgenic lines is due to the random gene integrations in the genome. This phenomenon is called position effect variation (Hansen and Wright 1999). Marchant et al. (1998b) reported that in rose, positional effects were more critical than transgene copy number in terms of gene expression.

All transgenic lines, in the present study, were able to grow on kanamycin medium giving an indication that gene silencing had not occurred in these shoots. However, phenotypical analysis of all transgenic lines in response to different stresses is required to confirm the stability of these lines. Further analysis of one of the selected transgenic lines (line 7, Fig 2) showed a few abnormalities in flowers of some regenerated plants. Green leaf-like organs observed in the centre of some flowers were due to deformed carpels. Also one flower with two ovaries was observed in one of the plants. These changes might be the result of somaclonal variation. Somaclonal variation can occur during regeneration, especially via indirect organogenesis and somatic embryogenesis. Usually, proliferation of embryogenic callus for long period causes somaclonal variation. For example, morphological abnormalities including thick and fleshy stems, abnormal leaves, high number of thorns, different petal numbers and shape, dwarf growth habit and different flower color have been reported in regeneration studies of some rose cultivars (Arene et al. 1993; De Wit et al. 1990; Lloyd et al. 1988).

Adventitious root induction on transgenic shoots was very difficult in this study. This problem can reduce the worthiness of these transgenic plants at high scale production. The lower rate of root induction on transgenic shoots might be due to the interaction between cytokinin, produced in transgenic plants under stress, and auxin of the media. Since the production of cytokinin in leaves starts under stress condition, reducing the stress such as root induction under mist or reducing leaf area might be useful in increasing the rooting frequency. However, under *in vitro* condition, removing leaves of shoots did not help in adventitious root induction in preliminary experiments. As the
reduction in adventitious root induction has been reported previously in \( P_{SAG12- IPT} \) transgenic petunias (Chang et al. 2003; Clark et al. 2004) it is important to check the rooting ability of the cuttings of the plants generated in the current study. 

*Since the phenotypical analysis has not begun yet, results of preliminary experiments with in vitro shoots under dark-stress are discussed in the general discussion of the thesis.*

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References


General Discussion
7. General discussion

Since the first report on somatic embryogenesis in rose about two decades ago (De Wit et al. 1990), several studies have been carried out with the aim of somatic embryo induction in rose cultivars from several types of roses including cut roses, garden roses and miniature roses. Therefore, several protocols are available which have worked successfully for distinct cultivars. However, most studies are either on garden roses or cut roses, and only few reports are available on miniature roses. Since the present work was carried out on miniature potted rose cultivars, the hypothesis was that:

- Regeneration of miniature-potted rose cultivars can be conducted using protocols established for other rose cultivars

It was assumed that available protocols without or with few modifications were useful for somatic embryogenesis in selected miniature-potted rose cultivars. However, after trying several available protocols on first selected cultivars (‘Linda’, ‘Leonie’ and ‘Rosita’), no useful results were obtained in preliminary experiments. Several factors including genotype, size and type of explant, plant growth regulators (PGRs), salts, carbohydrates, gelling agents and preculture conditions are important for somatic embryogenesis. Therefore, more modifications of the protocols were required to obtain embryogenic callus in the tested cultivars. Thus, six more cultivars of miniature roses were added to the experiments and also two garden rose cultivars from another lab (Ahrensburg, Germany) were used as control. Except for ‘Rosita’, which was omitted from the experiments, the rest of the cultivars (eight miniature rose cultivars) were included in a more comprehensive study (Zakizadeh et al. 2008). Final results showed that, in agreement with results of other researchers, somatic embryogenesis is highly genotype dependent and additionally a high degree of optimisation is needed for each cultivar. In agreement with results of previous studies in the miniature rose *R. chinensis minima* (Hsia and Korban 1996; Li et al. 2002), higher concentrations of auxins are required for callus production in these cultivars compared with garden rose cultivars. High levels of PGRs were required not only at the callus production stage, but also for regeneration and development of somatic embryos. These requirements might be due to lower levels of endogenous hormones in miniature roses, which cause the slower growth rate of these cultivars. Since *R. chinensis minima* is the ancestor of all miniature roses (Horn 1992), the lower hormonal levels, which could be under control of dominant genes, might have been inherited from this species. However, hormonal levels in miniature roses have not been measured yet and that is interesting to consider in future studies. In general, the
frequency of somatic embryogenesis in rose is very low and it is highly genotype
dependent. Schum and Dohm (2003) have reviewed somatic embryogenesis in rose and
reported that the development of regeneration protocols for each cultivar is still based on
trial-and-error approaches, which is time consuming and may not lead to successful
results. Another problem is that although embryogenic callus was reported in some
studies, successful recovery of flowering plants was not always possible. Abnormal
somatic embryos have been observed in many studies and often they failed to germinate.
Plant conversion (germination of somatic embryos) is one of the big challenges in
somatic embryogenesis in many species (Wann 1989). Therefore, in the present study
several cultivars have been used in the beginning of the experiment and after obtaining
promising results for some of them, the rest has been omitted from the experiment, and in
the end the study continued with only one cultivar. Since abnormality and low frequency
in plant conversion is mostly due to insufficient maturation of somatic embryos, ABA
was used for both multiplication and maturation of somatic embryos and also to increase
the germination frequency.

One of the problems regarding the breeding of miniature potted rose cultivars is that
cultivars do not remain on the market for a long period, and there is a high demand for
new cultivars of miniature potted roses. For example, most of the cultivars, which were
used for the present study, went out of the market soon after the experiments were begun.
On the other side, conventional breeding programs are time consuming and constrained
by some restrictions including gene pool limitations, high degree of inter-cultivar sterility
due to different ploidy levels, perennial nature of rose plants, high degree of
heterozygosity and lack of inbred lines. Several of these problems can be overcome by
making transgenic plants via biotechnological methods. However, these methods depend
on the ability of successful transformation at the cellular level and an efficient
regeneration system from the transformed cells (Hansen and Wright 1999; Li et al. 2002;
Robinson and Firoozabady 1993). Therefore, a better understanding of the processes
underlying these events is necessary for improving breeding programs and for developing
these methods to be used for any genotype of interest.

According to previous studies, embryogenic callus and somatic embryos are the best
explants for rose transformation. However, induction of somatic embryogenesis on
already established cultivars in order to introduce a new gene and regeneration of plants
is a time consuming process. For example, producing transgenic plants in the present
study took almost four years and most of the time was spent on induction of somatic
embryos and regeneration of transgenic shoots. Therefore, to remove this obstacle and to
speed up the process of somatic embryogenesis in new cultivars, it is of great importance to be able to distinguish the cultivars, which are able to go through somatic embryogenesis, at an early stage. In this regard, investigations were initiated to identify genes, which possibly have a role in somatic embryogenesis. The aim of these studies was to find a reliable molecular marker for recognizing callus, which is competent to develop into the embryogenic condition (Chugh and Khurana 2002; Feher et al. 2003). Several studies with different plants have been performed and they ended up with discovery of a somatic embryogenesis receptor like kinase (SERK) gene in Daucus carota (Schmidt et al. 1997). The DcSERK gene can be used as a reliable molecular marker for recognizing callus, which is competent to go through somatic embryogenesis from non-embryogenic callus. More studies resulted in isolation of SERK homologues from different plant species. These studies have shown that homologues of SERK genes from Daucus carota, Arabidopsis thaliana and Medicago truncatula can be used as a reliable molecular marker for this purpose (Hecht et al. 2001; Schmidt et al. 1997; Somleva et al. 2000). Therefore, it was hypothesized that:

- Homologue(s) of somatic embryogenesis receptor-like kinase (SERK) gene(s) exist in rose species

Thus, related degenerate primers were designed using SERK sequences from other species and used for screening of rose genomic DNA for isolation of possible SERK homologues (Zakizadeh et al. paper 2). The results revealed the existence of four SERK homologues in rose species. The partial sequences of the rose SERK genes were designated as RhSERK1-4 and submitted to the NCBI GenBank (Appendix). Phylogenetic trees constructed using nucleotide (Fig. 5-3, page 61) and protein (Fig. 3, page 82) sequences showed the close relationship among SERK sequences from rose and from other species. For example, at nucleotide level RhSERK1 is very similar to Arabidopsis SERK sequences (AtSERK4-5) and RhSERK4 is very similar to rice SERK (OsSERK1). At amino acid level, RhSERK1 and AtSERK3, as well as RhSERK3 and TcSERK are very similar. Since it has been reported that SERK genes from Arabidopsis, rice and cacao have a role in somatic embryogenesis, it was supposed that:

- SERK gene(s) from rose have a role in somatic embryogenesis and can be used as a marker for distinguishing cells which are competent to form somatic embryos in in vitro culture

Therefore, total RNA extracted from petals, stamens, in vitro grown leaves, non-embryogenic callus, repetitive embryogenic callus, matured embryos (cotyledonary
stage) and *in vitro* roots of *R. hybrida* ‘Linda’ was used to produce cDNA. This cDNA was amplified in a semi-quantitative RT-PCR reaction by means of specific primers and an expression analysis of the *RhSERK* genes was conducted. The results showed that *RhSERK* genes are expressed in all tested tissues including both embryogenic and non-embryogenic ones (Zakizadeh et al., paper 2). Expression of these genes in embryogenic callus proved the role of *RhSERK* genes in this phenomenon; however, expression in non-embryogenic tissues may indicate a wider role for these genes in rose species. These results are in agreement with results of previous studies on SERK homologous in other species including rice (Ito et al. 2005), maize (Baudino et al. 2001) and *Citrus unshiu* (Shimada et al. 2005). Therefore, these rose SERK genes are not reliable markers for this purpose. However, this study provided useful information about these genes and their activity in some tissues of rose. More studies on these genes including isolation of complete *RhSERK* sequences, isolation and characterization of their promoters, making P<sub>RhSERK</sub>-GUS (or P<sub>RhSERK</sub>-GFP for non-destructive measurements) and P<sub>35S-RhSERK</sub> constructs and further transformation of model plants with these constructs could produce more information and better understanding of rose SERKs. Moreover, expression analysis of *RhSERK* genes using Real-time RT-PCR on different tissues and also different parts of seeds especially zygotic embryos may provide more interesting results about these genes.

As has been mentioned before, modern breeding programs using biotechnological methods can overcome many of the problems existing in conventional breeding programs. Several studies with *Agrobacterium*-mediated transformation have been carried out in different rose cultivars and most of them ended up with successful results (Chapter 4). Several protocols are available for transformation. In contrast to somatic embryogenesis, which is highly genotype dependent and needs high degree of optimization, transformation protocols are rather similar to each other and therefore it was supposed that:

- *Transformation of rose somatic embryos with the P<sub>SAG12-IPT</sub> construct can be achieved using Agrobacterium tumefaciens strains*

In general, the genetic transformation of plants depends on several factors such as plant species, cultivar, explant type, an appropriate selection system and transformation method. Plant transformation using *Agrobacterium* strains has become the most used method for introducing a new gene into plant cells (De la Riva et al. 1998; Slater et al. 2003). Some species are easy to transform while others such as many woody plants are
recalcitrant to transformation (De la Riva et al. 1998). Susceptibility to crown gall disease has a genetic basis in cucurbits, peas, soybeans, grapevines and even among various ecotypes of *Arabidopsis thaliana* (Gelvin 2003). Therefore, also in rose it can be very different from species to species and from cultivar to cultivar. Rose is a woody species and since the first transformation on this species 14 years ago (Firoozabady et al. 1994), less than 10 successful transformation studies have been reported using *Agrobacterium* strains (Chapter 4). In some cases successful transformation of explants is reported but recovery of transgenic plants has not been described (Derks et al. 1995). Moreover, only two reports of successful biolistic transformation of one rose cultivar (*R. hybrida* ‘Glad tidings’) are available (Marchant et al. 1998a; 1998b). Although transformation using *Agrobacterium* strains has advantages over the biolistic method, both methods were used in the present study. The biolistic method was used in order to save time and also as a back-up in case the *Agrobacterium*-mediated technique failed to give positive results. However, after getting negative results from two preliminary experiments, the biolistic method was not used any further. For both transformation methods, embryogenic callus containing matured somatic embryos was used as explants. With the *Agrobacterium*-mediated technique, a wide range of variables was tested in both preliminary and main experiments in order to increase the chances of success. Different *Agrobacterium* strains, different times of inoculation and cocultivation, different media for dilution of *Agrobacterium* culture and different antibiotics at various concentrations are some of these variables. Finally, results were obtained with one *Agrobacterium* strain and one of the treatments at low transformation frequency (Manuscript).

The usage of antibiotics is necessary in *Agrobacterium*-mediated transformation especially after cocultivation in order to prevent the overgrowth of *Agrobacterium* on explants. A kanamycin resistance gene usually exists in many constructs to make the selection of transgenic plants easier. However, during early stages of selection, the putative transgenic explants or shoots might not be resistant to high concentrations of selectable antibiotic such as kanamycin. For example, in the present study no transgenic shoots survived using kanamycin even at concentrations as low as 40 mg l\(^{-1}\) in the medium. Therefore, putative transgenic matured embryos were transferred to antibiotic-free medium and regenerated shoots were multiplied and then transferred to kanamycin containing medium for confirmation of transformation. One reason for this could be transformation of explants with parts of T-DNA that only has the PsAG12-IPT gene and not antibiotic resistant gene. However, in the present study, further PCR analysis of
putative transgenic shoots confirmed the existence of the whole cassette in all different lines (Manuscript).

In general, antibiotics have negative effects on proliferation and differentiation of plants (Li et al. 2002) and also have negative effects on the environment. Metabolic markers can be potential alternatives for antibiotic resistance genes. They enable the plants to grow on unfamiliar culture media or to produce metabolic products, which allow only the transgenic cells to grow. However, the marker genes are generally no longer needed once the transgenic cells have been successfully identified. Moreover, according to the new rules on genetically modified organisms (GMO), crops with antibiotic resistance genes and/or with general marker genes will not be allowed to be commercialized in the near future (European Parliament, 2001). For this reason, several studies are looking for different ways of achieving gene transfer without marker genes or the subsequent removal of the marker genes. One of the strategies for producing marker-free transgenic plants is use of transposable elements. For example, the ipt gene on a transposable element has been used as a selectable marker in transformation of tobacco and after moving the transposable element to a new location in the genome; a marker-free transgenic plant will be obtained which can be used for a second transformation (Ebinuma et al. 1997). Cocultivation with natural strains of A. rhizogenes has also been used for producing a marker-free dwarf plants in some species.

Another approach is co-transformation. The principle is based on delivery of two separate T-DNAs, one with genes of interest and the other with marker genes. The transgene and marker gene are often inserted into different sites in the plant genome. Therefore they can be separated during meiosis and plants, which carry only the transgene can then be selected (Hansen and Wright 1999). Since woody plants have a longer life compared to herbaceous plants, they can transfer the new genes to other plants or organisms during their long life. Therefore, more care need to be taken to prevent distribution of a new gene to the environment. Producing male sterile plants and also transformation of plastids instead of genomic DNA are of great interest. Chloroplast transformation is a new development in the 21st century and it offers opportunities for making GM crops, which are both efficient and environmentally friendly. Since chloroplasts in many species display only maternal inheritance, this method prevents transferring new genes to other plants via pollen. Additionally, there is no report of gene silencing with chloroplast transformation (Slater et al. 2003). These techniques might be able of use in transformation of rose cultivars in the future.
Discussion

Although most senescence retardation studies have worked with modulation of ethylene synthesis and/or perception, according to the literature, manipulation of ethylene is more useful in retardation of fruit and flower senescence than leaf senescence. On the other hand, several studies have shown that modulation of cytokinin levels is more useful for inducing significant delay of senescence in leaves than in fruits and flowers (Noh et al. 2004). Therefore, the chimeric gene P\textsubscript{SAG12}-IPT, which has been used successfully for senescence retardation in many species (Chapter 3), was used in the present study. After confirmation of transgenic shoots, different transgenic lines must be analysed. According to the literature, it was assumed that:

- Transgenic plants will exhibit significant delay in leaf senescence and will be, except for this characteristic, undistinguishable from control plants

Transgenic shoots were propagated on shoot multiplication medium (Zakizadeh et al., Paper 1) before they were transferred to the rooting medium. Transgenic shoots did not show any morphological abnormalities, however, they showed broader leaves and thicker stems compared to control shoots during the \textit{in vitro} stage. Since the SAG12 promoter should be only expressed at the start of senescence, differences between transgenic and control plants grown under similar \textit{in vitro} conditions indicated leakiness of the SAG12 promoter in rose (Manuscript). However, \textit{in vitro} condition might be considered as stress inducer. Leakiness of the SAG12 promoter in the P\textsubscript{SAG12}-IPT construct has been reported in other studies (Chang et al. 2003; Clark et al. 2004; Schroeder et al. 2001).

To analyse the plants, the propagated \textit{in vitro} shoots were transferred to rooting medium to produce plantlets for greenhouse experiments. At this stage, root induction on transgenic shoots was very difficult. The inhibition of root induction in IPT transgenic plants has been reported previously (Gan and Amasino 1996). But the P\textsubscript{SAG12}-IPT senescence retardation system is designed as an autoregulatory senescence-inhibition system, and induction of the SAG12 promoter should ideally only take place when senescence starts in leaves and other green tissues of the plant. However, rooting experiments with transgenic shoots resulted in high callus production instead of root induction. These responses might indicate the effects of low nutrients in rooting medium in initiation of senescence and consequently induction of the SAG12 promoter. These results are in agreement with results of previous studies where low levels of nutrients were used for senescence induction in P\textsubscript{SAG12}-IPT transgenic plants (Jordi et al. 2000; McCabe et al. 2001; Schroeder et al. 2001). Removing leaves, which were supposed to be the main center of cytokinin production in these transgenic plants, did not help to reduce
the callus production in the rooting medium. It seemed that cytokinin is produced in both leaves and stems under low nutrients stress and translocated to the basipetal end of shoots and that the changed auxin/cytokinin ratio lead to production of undifferentiated callus.

Another reason for high callus production in the rooting media might be the use of high levels of IBA plant growth regulator in the rooting medium. IBA has the tendency to induce excessive callus production in tissue culture (S. Sriskandarajah, personal communication). After reducing the IBA levels and increasing IAA concentrations, roots were produced in some of the transgenic shoots (Manuscript). However, high levels of IBA did not induce callus production on control shoots. Reduced root formation has previously been reported in P_{SAG12}-IPT transgenic petunias (Chang et al. 2003; Clark et al. 2004). In general, this is one of the disadvantages of these transgenic shoots and this characteristic may reduce the value of these plants for commercial production. However, this was particularly the behavior of in vitro grown shoots and not of the cuttings from greenhouse grown plants. Therefore, it is required to compare the rooting efficiency in cuttings from greenhouse grown plants of both transgenic and control plants with and without root induction treatments.

For analyzing the effects of new traits of the transgenic plants it is of importance to expose them to different stress conditions such as darkness, drought, low nutrients and even exposure to ethylene and observe their reaction compared to control plants. However, due to the time limit of the project, greenhouse experiments were postponed to the future. Instead, an experiment with in vitro grown shoots was carried out exposing both transgenic and control shoots to dark-stress. However, some results of the rooting experiments can be considered as low nutrient-stress because some of transgenic shoots remained green after six months on low nutrient-rooting medium without sub-culturing to the fresh medium (Fig. 7-3.b). For dark-stress, shoots were cultured in containers with shoot multiplication medium and kept in the dark for 24 days in a growth room at 24±2°C. These cultures were checked after 2, 4, 7, 9, 11, 15, 22 and 24 days to compare the reaction of transgenic and control shoots to dark-stress and senescence processes (Fig. 7-1). After 24 days cultures were returned to light under the same general growth conditions to see how much they recovered. The results showed that senescence started in non-transformed shoots after 4 days under darkness, while transgenic shoots did not show any sign of senescence at the same time (Fig. 7-1). Although a few yellow leaves were observed in only 2-3 shoots of transgenic plants at day seven, these were new leaves grown in the absence of light and not results of stress. But, at the same time, senescence could be observed in almost all control shoots. The number of senescing leaves increased
in all shoots of control plants at day 11, while about half of the transgenic shoots in each container did not have any senescing leaves.

Figure 7-1. Effect of darkness on *in vitro* grown shoots of transgenic and control plants 4, 7, 15 and 24 days after stress. DADS: days after dark-stress; C: Control plants (Three replications); T: Transgenic plants (Three replications).
Discussion

After two weeks of dark-stress (day 15), several green shoots with green leaves could still be seen in transgenic plants, while in control plants, senescing leaves were dominant and very few green leaves could be observed. These results showed that, under dark-stress, senescence was delayed up to 10 days in \( \text{P}_{\text{SAG12}} \)-IPT transgenic plants compared to non-transformed plants of this miniature potted rose cultivar. Delaying senescence using the \( \text{P}_{\text{SAG12}} \)-IPT gene has been seen in previous studies in other crops (Chang et al. 2003; Chen et al. 2001; Clark et al. 2004; Gan and Amasino 1995; Jordi et al. 2000; McCabe et al. 2001; Schroeder et al. 2001).

At the end of dark treatment (day 24), most of the leaves of both control and transgenic plants had senesced. The difference between senescence of transgenic plants and control plants is that leaf senescence starts from old leaves which turn yellow in control plants, as is the case in naturally grown plants, while in the \( \text{P}_{\text{SAG12}} \)-IPT transgenic plants senesced leaves were more dark green. However, a few yellow to white leaves could be seen in transgenic plants, but these were new leaves which had grown in the absence of light (Fig. 7-2). The reason might be that the translocation of N takes place from older leaves to the younger ones in control plants but does not occur in transgenic plants during senescence. Chloroplasts are the first organelles that are broken down during leaf senescence and they contain up to 70% of the leaf protein and consequently a large portion of leaf N (Gan and Amasino 1996 and 1997; Thomas et al. 2003). Therefore, natural leaf senescence starts with breakdown of chloroplasts and translocation of N from older to younger leaves. However, Jordi et al. (2000) showed that in \( \text{P}_{\text{SAG12}} \)-IPT transgenic tobacco plants, it does not happen in the same way. Compared to wild-type, accumulation of N in transgenic tobacco plants was higher in senescing leaves than in younger leaves, leading to an inverted canopy N profile in these plants. High levels of N in old leaves were due to high cytokinin levels which consequently increased sink activity in these leaves for N (Jordi et al. 2000). High levels of N and consequently protein in older leaves showed the presence of healthy chloroplasts in those leaves. This phenomenon has been observed in petunia (Clark et al. 2004) and lettuce (McCabe et al. 2001) where chlorophyll content in lower leaves of \( \text{P}_{\text{SAG12}} \)-IPT transgenic lines were higher than in control plants. Therefore, the same phenomenon might have occurred in the transgenic roses causing increased postharvest longevity for about 10 days compared to control plants (Fig. 7-1).

In general, rose plants require high light intensity for optimum growth and performance. Potted plants are usually kept indoors, and under non-optimal conditions where enough light is not available and adequate care cannot be taken, these transgenic
Discussion

Plants might stay green for a longer period compared to non-transformed plants. Although other stress treatments such as drought-stress, low nutrient-stress and ethylene exposure were not applied, some results from the rooting experiments indicated improved postharvest performance of these plants under non-optimal conditions. Some of the shoots, which were subcultured on rooting media remained green after six months without sub-culturing to fresh media (Fig 7-3.b). However, more experiments with greenhouse-grown plants are required to support these preliminary results. Extraction and measurements of cytokinins for their types and amounts during senescence are also required. Expression analysis of the *ipt* gene in leaves of transgenic plants during the stress period will also be useful.

![Control shoots - Day 7](image1) ![Transgenic shoots - Day 11](image2)

Figure 7-2. Difference between leaf senescence in control plants and *P*SAG12-IPT transgenic plants under dark-stress. Leaves turned yellow in control plants while they remained green or dark green in transgenic plants after dark stress. The reason is assumed to be chloroplast breakdown and nitrogen translocation.

In general, apart from lower adventitious root induction compared to control shoots, these transgenic plants are interesting for use in production of new miniature cultivars of rose with better postharvest performance compared to non-transformed plants. However, since according to new rules on GM plants, antibiotic resistance genes are not allowed in the near future and actually marker free transgenic plants are of great interest, these transgenic plants cannot be used in breeding programs. They can be used for research purposes only to elucidate the effect of the *P*SAG12-IPT gene in increasing the postharvest life of rose species exposed to different stresses. If the transgenic plants obtained in this study seem valuable for rose breeding programs, a new marker-free construct with the same gene should be produced and used for transformation of rose cultivars.
Discussion

For future approaches and the construction of a new marker free \( P_{SAG12} \)-IPT gene, some modifications can be considered. At the time the used construct was made (1993), IPT genes from plant species have not been discovered, and therefore the \( ipt \) gene from \( A. \ tumefaciens \) was used for this construct and it worked successfully in plants. Today, several homologues of \( ipt \) gene have been isolated from \( A. \ thaliana \) (Kakimoto 2003; Takei et al. 2001) and have been characterized (Miyawaki et al. 2004 and 2006; Sun et al. 2003). Therefore, these genes might act better than the \( ipt \) gene from \( Agrobacterium \) in rose. However, it might be possible to isolate homologues of \( Arabidopsis \) IPT genes and also homologues of the \( SAG12 \) promoter from rose plants and use them for making a homologous \( P_{SAG12} \)-IPT gene construct for transformation of rose cultivars. A homologous construct should only work in the tissues where rose \( P_{SAG12} \) works and might lead to safer GM plants regarding the environment. Chloroplast transformation and also production of male sterile roses should also be considered.

Figure 7-3. Interactions between transgenic shoots and rooting media. a) A transgenic shoot two months after subculturing on rooting media containing low levels of IAA and high levels of IBA. b) Transgenic shoots remained green after six months growing on rooting medium containing high level of IAA hormone.

Future work

In summary, the following work can be suggested for future research:

1. Using techniques of molecular biology such as differential display and microarrays in non-embryogenic and embryogenic tissues of rose for the
discovery of genes which have specific role(s) in initiation of somatic embryogenesis and can be used as reliable molecular markers.

2. Isolation of complete RhSERK sequences, isolation and characterization of their promoters, making of different constructs (such as P_{RhSERK}-GUS, P_{RhSERK}-GFP and P_{35S}-RhSERK) and transformation of model plants with these constructs will be useful for better understanding of rose SERK sequences.

3. Expression analysis of RhSERK genes using Real-time RT-PCR on different tissues and also different parts of seeds, especially zygotic embryos may provide additional interesting information about these genes.

4. *In vitro* adventitious root induction in different transgenic lines and analysing greenhouse grown plants under different stress conditions such as dark- and drought-stress.

5. Comparing rooting of cuttings of both transgenic and control plants from greenhouse grown plants.

6. Grafting of transgenic plants on non-transformed plants as rootstock and characterization of the resulting plants.

7. Analyzing male fertility of different transgenic lines to see if they can be used for further breeding programs.

8. Analyzing the effects of exposing different lines of transgenic plants and control plants to different concentrations and exposure times of ethylene.

9. Extraction and measurement of the amounts of different types of cytokinins in these plants after exposure to different stress conditions.

10. Constructing a marker free P_{SAG12-IPT} gene for transformation of plants with same promoter and gene as used in the present study or alternatively using their homologues from rose.

11. Induction of somatic embryogenesis on leaves of transgenic plants to see whether the change in endogenous hormonal balance can affect frequency of somatic embryogenesis.
REFERENCES
References


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References


References


References


Appendix
Appendix

LOCUS       EF631967                 660 bp    DNA     linear   PLN 10-SEP-2007
DEFINITION  Rosa hybrid cultivar somatic embryogenesis receptor-like kinase 1 (SERK1) gene, partial cds.
ACCESSION   EF631967
VERSION     EF631967.1 GI:149346188
KEYWORDS    .
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ORGANISM    Rosa hybrid cultivar
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            Spermatophyta; Magnoliophyta; eudicotyledons; rosid; eurosids I;
            Rosales; Rosaceae; Rosoideae; Rosa.
REFERENCE   1  (bases 1 to 660)
AUTHORS     Zakizadeh,H., Stummann,B.M. and Mueller,R.
TITLE       Isolation and characterization of somatic embryogenesis receptor-like kinase (RhSERK) genes in miniature potted rose (Rosa hybrida cv. Linda)
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 660)
AUTHORS     Zakizadeh,H., Stummann,B.M. and Mueller,R.
TITLE       Direct Submission
JOURNAL     Submitted (24-MAY-2007) Agricultural Sciences, Faculty of Life Sciences, University of Copenhagen, Hojbakkegaard Alle, 21, Taastrup, DK 2630, Denmark
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LOCUS       EF631968                 595 bp    DNA     linear   PLN 20-JUN-2007
DEFINITION  Rosa hybrid cultivar somatic embryogenesis receptor-like kinase 2 (SERK2) gene, partial cds.
ACCESSION   EF631968
VERSION     EF631968
KEYWORDS    .
SOURCE      Rosa hybrid cultivar
ORGANISM  Rosa hybrid cultivar
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; rosids; eurosids I; Rosales; Rosaceae; Rosoideae; Rosa.
REFERENCE   1  (bases 1 to 595)
AUTHORS   Zakizadeh,H., Stummann,B.M. and Mueller,R.
TITLE     Isolation and characterization of somatic embryogenesis receptor-like kinase (RhSERK) genes in miniature potted rose (Rosa hybrid cv. Linda)
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 595)
AUTHORS   Zakizadeh,H., Stummann,B.M. and Mueller,R.
TITLE     Direct Submission
JOURNAL   Submitted (24-MAY-2007) Agricultural Sciences, Faculty of Life Sciences, University of Copenhagen, Hojbakkegaard Alle, 21, Taastrup, DK 2630, Denmark
FEATURES             Location/Qualifiers
source          1..595
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    /db_xref="taxon:128735"
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mRNA            join(<1..109,313..>595)
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    /product="somatic embryogenesis receptor-like kinase 2"
CDS             join(<1..109,313..>595)
    /gene="SERK2"
    /note="protein kinase"
    /codon_start=1
    /product="somatic embryogenesis receptor-like kinase 2"
    /protein_id="ABR23656"
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    /product="somatic embryogenesis receptor-like kinase 2"
    /protein_id="ABR23656"
    /translation="AVHRNLLRLRFGCMTPTERLLVYPYMANGSVASCLRERPPSQP"
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61 ctgcttata ctatatggc taatggttagc gttgctctct gtttaaggg tagaaagtgc
121 ttctgcatct tggatatctt ttgctctttt ttctctctct ttggttcttt ttaaatcttct
tttgttttt ctgctgtttt ttttctcatt tgtgggtttt aatggagtct tgttttagtt tttgccttct
181 tttgtgtttt tgtgttttct tatttctgca tgtgtttttt ataatgtttt cttctcttct
tgtaattttg tttcggagag agttcttttt ttttgtgtgt tatttattgtata cccttcttctt
241 ttttcgactt ttttcgctttt tcgtcttttt cttctcttcttt cttctctctt tttctctcttt
301 tgttttcttt tgggttctttt tcctctcttt ctctctctctt ctctctctctt cttctctctctt
ttttttcttt cttctctcttt cttctctcttt cttctctcttt cttctctcttt cttctctcttt
361 ttgcttgttttt tgtgtgttttt tgtgtgtgttttt tgtgtgtgttttt tgtgtgtgttttt tgtgtgtgttttt
ttttttttgtt ttttttttttt ttttttttttt ttttttttttt ttttttttttt ttttttttttt
421 tgccttat cttctttttt tgtctttttt tgtctttttt tgtctttttt tgtctttttt
tgtctcttttt tgtctcttttt tgtctcttttt tgtctcttttt tgtctcttttt tgtctcttttt
481 gacacattt cttttttttttt ttttattgtttt ttttattgtttt ttttattgtttt ttttattgtttt
541 tgttttttt cttctttttt cttctttttt cttctttttt cttctttttt cttctttttt

//
Appendix

LOCUS EF631969  381 bp  DNA  linear  PLN 10-SEP-2007
DEFINITION Rosa hybrid cultivar somatic embryogenesis receptor-like kinase 3 (SERK3) gene, partial cds.
ACCESSION EF631969
VERSION EF631969.1  GI:149346229
KEYWORDS .
SOURCE Rosa hybrid cultivar
ORGANISM Rosa hybrid cultivar
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; eudicotyledons; rosids; eurosids I; Rosales; Rosaceae; Rosoideae; Rosa.
REFERENCE 1  (bases 1 to 381)
AUTHORS Zakizadeh,H., Stummann,B.M. and Mueller,R.
TITLE Isolation and characterization of somatic embryogenesis receptor-like kinase (RhSERK) genes in miniature potted rose (Rosa hybrid cv. Linda)
JOURNAL Unpublished
REFERENCE 2  (bases 1 to 381)
AUTHORS Zakizadeh,H., Stummann,B.M. and Mueller,R.
TITLE Direct Submission
JOURNAL Submitted (24-MAY-2007) Agricultural Sciences, Faculty of Life Sciences, University of Copenhagen, Hojbakkegaard Alle, 21, Taastrup, DK 2630, Denmark
FEATURES Location/Qualifiers
source          1..381
    /organism="Rosa hybrid cultivar"
    /mol_type="genomic DNA"
    /db_xref="taxon:128735"
gene            <1..>381
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mRNA            join(<1..19,218..>381)
    /gene="SERK3"
    /product="somatic embryogenesis receptor-like kinase 3"
CDS             join(<1..19,218..>381)
    /gene="SERK3"
    /note="protein kinase"
    /codon_start=1
    /product="somatic embryogenesis receptor-like kinase 3"
    /protein_id="ABR23657.2"
    /db_xref="GI:157062428"
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BASE COUNT       83 a     79 c     77 g    142 t
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61 ttctgtgagg gctgctttct ttgtcttgtt ggtttctgct tgtttatttc tagcagtggt
121 tttctaaatg tcatttctcc catctttgca aattttttca gaaggattta cttttctgcg
181 tttgttaattt atgcatacct tttatgctgt cctttagaac gcgcaccatc ccaaccacct
241 ctttgacctgcc caacctcgaa gcgaacctgga cttgggatctg cgagggcgct tttttatttg
301 cacagactc gtgaccaccaaa gattattcacc ctgtgatgta aagctgcaa ctatcgtgct
361 gatgaggaat tcgaagccat g

Appendix

LOCUS       EP631970                 404 bp    DNA     linear   PLN 20-JUN-2007
DEFINITION  Rosa hybrid cultivar somatic embryogenesis receptor-like kinase 4
(SERK4) gene, partial cds.
ACCESSION   EP631970
VERSION     EP631970
KEYWORDS    .
SOURCE      Rosa hybrid cultivar
  ORGANISM  Rosa hybrid cultivar
         Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
         Spermaphyta; eudicotyledons; core eudicotyledons; rosids; eurosids I;
         Rosales; Rosaceae; Rosoideae; Rosa.
REFERENCE   1  (bases 1 to 404)
  AUTHORS   Zakizadeh,H., Stummann,B.M. and Mueller,R.
  TITLE     Isolation and characterization of somatic embryogenesis
         receptor-like kinase (RhSERK) genes in miniature potted rose (Rosa
         hybrid cv. Linda)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 404)
  AUTHORS   Zakizadeh,H., Stummann,B.M. and Mueller,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-MAY-2007) Agricultural Sciences, Faculty of Life
         Sciences, University of Copenhagen, Hojbakkegaard Alle, 21,
         Taastrup, DK 2630, Denmark
FEATURES             Location/Qualifiers
  source          1..404
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         /mol_type="genomic DNA"
         /db_xref="taxon:128735"
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  mRNA            join(<1..22,134..>404)
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         /product="somatic embryogenesis receptor-like kinase 4"
  CDS             join(<1..22,134..>404)
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         /note="proteiin kinase"
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         /product="somatic embryogenesis receptor-like kinase 4"
         /protein_id="ABR23658"
         /translation="SVASRLKSKPALDWGTRKRIAAGARGLYLHEQCDPKIIHRDV
         KAANILDYFEAVVGDGLAKPLLHHEHITTTAVRTGVHATAPEYLTSTQCSS"
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   61 ttaatcacta ctttatatta attcctttgc ctttagaact agtgaatgaa cacaaacttt
  121 gcagcaaggg gacttctgta cctgcatgag cagtgtgacc ccaagatcat tcatagggat
  181 gtaaaggctg caaatatact gcttgatgat tactttgagg ctgtggtggg agattttggg
  241 tttggcaaaac ttttggatca ccatgaatca cacattacaa cagcggtgag gggcaccgtg
  301 gggcacatag caccggagta tctctcaact ggccagtcct cgga
  361 gggcaacatag ccaccgggtg tctctcaact ggccagtcct cgga
//
LOCUS       EF631971                 264 bp    DNA     linear   PLN 20-JUN-2007
DEFINITION  Rosa hybrid cultivar actin gene, partial cds.
ACCESSION   EF631971
VERSION     EF631971
KEYWORDS    .
SOURCE      Rosa hybrid cultivar
ORGANISM  Rosa hybrid cultivar
          Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
          Spermatophyta; Magnoliophyta; eudicotyledons; rosids; eurosids I; Rosales; Rosaceae; Rosoideae; Rosa.
REFERENCE   1  (bases 1 to 264)
AUTHORS   Zakizadeh,H., Stummann,B.M. and Mueller,R.
TITLE     Isolation and characterization of somatic embryogenesis
          receptor-like kinase (RhSERK) genes in miniature potted rose (Rosa
          hybrida cv. Linda)
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 264)
AUTHORS   Zakizadeh,H., Stummann,B.M. and Mueller,R.
TITLE     Direct Submission
JOURNAL   Submitted (24-MAY-2007) Agricultural Sciences, Faculty of Life
          Sciences, University of Copenhagen, Hojbakkegaard Alle, 21,
          Taastrup, DK 2630, Denmark
FEATURES             Location/Qualifiers
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          CDS             <1..>264
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                                      EQELEAAASSSTIEKDYELPDQVITIGAERFRCPEVLFQPSLV"
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1 cttgcrtggac gagatattaac agacctccta atgaagatcc tgactgagag agggtattct
61 tttactacaa cttgcrtgac ggaatttga cttgcatatta aagagaagct tgcatatctg
121 gcacttgatt atgaacaaga aacttgagact gctgcaagca gctccacaat tgagaaggac
181 tatgaactac ctggatggtca ggtaattacc attggagcag agcgtttccg ttgccctgaa
241 gtcctatttc aaccatcact agtt
//