Leaf senescence in silver birch (*Betula pendula* Roth)

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ABBREVIATIONS

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
Adh	alcohol dehydrogenase
Aos	allene oxidase synthase
APX	ascorbate peroxidase
Atub	alpha tubulin
BA	benzylaminopurine
Ca	carbonic anhydrase
Cab	chlorophyll a/b binding protein
Cat	catalase
cDNA	complementary DNA
Chs	chalcone synthase
Cyp	cysteine protease
Dad	defender against apoptotic cell death
DHAR	dehydroascorbate reductase
Dhn	dehydrin
DS3	3-deoxy-D-arabinoheptulosonate-7-phosphate
DOY	day of year
ein	ethylene-insensitive
est	expressed sequence tag
Etr	ethylene receptor
GA	gibberellin
GPX	glutathione peroxidase
G6PD	glucose-6-phosphate dehydrogenase
GR	glutathione reductase
Gst	glutathione-S-transferase
GUS	β-glucuronidase
ipt	*iso*-pentenyl transferase
JA	jasmonate
Lap	leucine aminopeptidase
LOX	lipoxygenase
LSU	large subunit of Rubisco protein
npt	neomycin phosphotransferase
NR	never ripe
MDAR	monodehydroascorbate reductase
Mpt	mitochondrial phosphate translocator
mRNA	messenger RNA
Pal	phenylalanine ammonium lyase
PR	pathogenesis-related
PSII	photosystem II
rbcL	large subunit of Rubisco gene
RbcS	small subunit of Rubisco gene
R/FR	red/far-red
RH	relative humidity
ROS	reactive oxygen species
SAG	senescence-associated gene
SAM	*S*-adenosyl-L-methionine
SDG	senescence-downregulated gene
sid	senescence-induced deficiency
SEN	senescence-associated gene
SEE	senescence-enhanced gene
SENU	senescence-upregulated gene
SOD	superoxide dismutase
SSU	small subunit of Rubisco protein
TDZ	thidiazuron
WRKY	DNA-binding protein containing amino acid sequence WRKYGCQ
YLS	yellow-leaf specific
yfd	youngest fully developed
Ypr10	pathogenesis-related protein 10 gene
QTL	quantitative trait loci

ORIGINAL PUBLICATIONS

This thesis is based on the following publications and a manuscript, which are referred to in the text by their Roman numerals. Additional unpublished data are also presented in the text.

I **Valjakka M, Luomala E-M, Kangasjärvi J and Vapaavuori E 1999.** Expression of photosynthesis- and senescence-related genes during leaf development and senescence in silver birch (*Betula pendula* Roth) seedlings. Physiologia Plantarum 106: 302-310.

II **Valjakka M, Aronen T, Kangasjärvi J, Vapaavuori E and Häggman H 2000.** Genetic transformation of silver birch (*Betula pendula* Roth) by particle bombardment. Tree Physiology 20: 607-613.

III **Sillanpää M, Luomala E-M, Vapaavuori E, Sutinen S, Kangasjärvi J, and Häggman H.** Characterisation of leaf senescence in silver birch (*Betula pendula*) with the help of a late–senescing mutant. Manuscript.

IV **Ryynänen L, Sillanpää M, Kontunen-Soppela S, Tiimonen H, Kangasjärvi J, Vapaavuori E, Häggman H 2002.** Preservation of transgenic silver birch (*Betula pendula* Roth) lines by means of cryopreservation. Molecular Breeding 10: 143-152.

SUMMARY

Leaf development from young expanding leaves to advanced senescence was followed in silver birch seedlings (*Betula pendula* Roth) during the first growing season and in young trees, representing clones V5952 and K1659, during the sixth growing season. In addition, leaf ontogeny was studied in a late senescing line, R3.1, which was found among transgenic lines produced in the present study. The leaves of line R3.1 were compared to leaves of wild-type clone R. Leaf developmental stages, young, mature and senescing, were determined by changes in photosynthetic activity based on maximal and actual efficiency of PSII, and the amount and activity of Rubisco enzyme. Photosynthetic activity increased with leaf expansion and started to decrease in fully expanded leaves. New information was gained on leaf senescence-related changes in silver birch; downregulation of photosynthesis and remobilization-related changes such as protein degradation and changes in gene expression level were well coordinated. Characteristic genes of leaf senescence such as cysteine protease (*Cyp*) and pathogenesis-related (PR) protein genes (*PR1*, *PR3a*, *Ypr10*) were also identified, and a few novel senescence-associated genes such as mitochondrial phosphate translocator (*Mpt*) and leucine aminopeptidase (*Lap*) were also found. Senescence-related changes were detected soon after leaves were fully expanded in silver birch seedlings, and also clone R, which were propagated for their first growing season under greenhouse conditions, whereas no senescence-related changes were observed in the insertion mutant line R3.1. The fully expanded leaves of field-grown 6 year-old silver birches maintained active photosynthesis for several weeks before they started to senesce.

A genetic transformation method was developed for silver birch in order to study regulation of carbon metabolism in transgenic lines containing reduced Rubisco enzyme, which is the key enzyme of carbon metabolism. For further studies, several sense-*RbcS* lines were produced from two silver birch clones, R and E5396, by a biolistic transformation method. As a long-term solution for maintenance of transgenic silver birch lines, a cryopreservation method was applied to cold-hardened buds of transgenic silver birch plants. After cryostorage, buds were thawed and regenerated plants were used to study the effect of freezing to neomycin phosphotransferase (*nptII*) transgenes. Cryopreservation treatment affected neither the stability nor the expression of *nptII* genes, and it is therefore a suitable maintenance method for transgenic silver birch lines.

1 INTRODUCTION

1.1 Types of leaf senescence in plants

Senescence is the final stage in the life span of a leaf, and leads to death and abscission. Leopold (1980) has classified different types of leaf senescence based on when and which part of the plant senesces. When a whole plant, such as an annual cereal, senesces due to reproductive development, this is called ‘monocarpic senescence’. In plants forming bulbs and tubers, only the parts which are above ground are actually dying. This is called ‘top senescence’. Leaf senescence in broad-leaved trees at the end of the growing season is called ‘deciduous senescence’, which is often referred to as ‘autumnal senescence’ (Smart 1994). In the ‘progressive senescence’ seen in annual and perennial plants, the oldest leaves die first. This is also referred as ‘sequential senescence’.

1.2 Changes during leaf senescence

Senescence is controlled by nuclear genes (Smart et al. 1995, Noodén et al. 1997). New proteins are synthesised, for example, to mediate degradation and transport, and to maintain cell metabolism. At the whole plant level, the main purpose of leaf senescence is to enable nutrient recycling, especially of nitrogen. Nitrogen is translocated from the ageing leaves, where photosynthetic activity has decreased, to storage tissues for the next growing season, or to the new developing tissues. This gradual catabolism requires and causes major changes in cell function.

1.2.1 Structural and functional changes at the cellular level

Various methods have been applied in studies aimed at understanding how senescence proceeds within leaves and cells. Generally, senescence starts in distant parts of the leaves, and the cells near the vascular bundle are the last ones to senesce. In cells, senescence-related changes are first detected in the chloroplast (Dodge 1970), whereas the mitochondria and nucleus remain intact until advanced senescence (Woolhouse 1984, Smart 1994, Inada et al. 1998). The changes observed within cells are presented in Table 1, and these changes will be described in detail in the following chapters.

Table 1. The changes observed in cells during leaf senescence.

Location	Change	Reference
Nucleus	- chromatin condensation	O'Brien et al. 1998
	- DNA fragmentation	Yen and Yang 1998
Chloroplasts	- DNA and RNA degradation	Inada et al. 1998, Bate et al. 1991
	- degradation of thylakoid membranes	Dodge 1970 Takamiya et al. 2000
	- pigment degradation	Viestra 1996
	- protein degradation	Biswal et al. 1983, Dodge 1970
	- number and size of plastoglobuli increase	
Mitochondria	- the number of mitochondria increases	Pastori and del Río 1994 Solomos 1988, Oleksyn et al. 2000
	- respiration increases	
	- production of ROS increases	Jiménez et al 1998
Peroxisome	- the number of peroxisomes increases	Pastori and del Río 1994 Pastori and del Río 1994, Landolt and Matile 1990
	- some peroxisomes are converted to glyoxysomes	Pastori and del Río 1994
	- production of ROS increases	Pastori and del Río 1994
	- peroxidation of lipids	Distefano et al. 1999
	- protein degradation	
Vacuole	- degradation of proteins, pigments	Viersta 1996, Takamiya et al. 2000
Cytosol	- amount of RNA decreases	Lohman et al. 1994, Maslaux et al. 2000

1.2.1.1 Chloroplasts

Chloroplasts contain the major proportion of cellular nitrogen, up to 70-80% (Makino and Osmond 1991), and most of this is bound to photosynthesis-related proteins. The volume of chloroplasts decreases, their shape turns from oval to rounded, the size or the number of plastoglobuli increases, and thylakoids disappear in naturally senescing birch leaves (Dodge 1970) and in *Sinapis alba* L. (mustard) cotyledons during dark-induced senescence (Biswal et al. 1983). In *Oryza sativa* (rice) coleoptiles that have a life span of only one week, senescence proceeds within a few days (Inada et al. 1998). The shrinking of chloroplasts, and the degradation of Rubisco and the inner chloroplast membrane follow degradation of DNA. Degradation of chlorophyll (Takamiya et al. 2000) and Rubisco (Desimone et al. 1998) starts in chloroplasts, but further degradation may occur outside of the chloroplasts, since plastoglobuli secreted or blebbed out from the chloroplast (Guiamét et al. 1999) contain chlorophyll, carotenoids and photosynthesis-related proteins (Guiamét et al. 1999) as well as lipid metabolites derived from thylakoids (Thompson et al. 1998).

1.2.1.2 Energy production

During leaf senescence, the decrease in photosynthetic activity is characterized by a decrease in the light-harvesting capacity of PSII (Humbeck et al. 1996, Lu and Zhang 1998), the amount and activity of Rubisco (Batt and Woolhouse 1975, Jiang et al. 1993), CO_2-exchange rate (Jiang et al. 1993), and the level of mRNA expression of photosynthesis-related genes (Bate et al. 1991, Humbeck et al. 1996). When photosynthesis decreases during senescence, chloroplasts produce less NADPH and ATP. However, energy is needed for catabolic processes and it has been suggested that increasing respiration detected both in detached and attached leaves during senescence would provide ATP for these processes (Solomos 1988, Oleksyn et al. 2000). The number of mitochondria was reported to increase in *Pisum sativum* L. (pea) leaves during dark-induced senescence (Pastori and del Río 1994). Also, the number of leaf peroxisomes increases (Pastori and del Río 1994). Some peroxisomes may be converted to glyoxysomes which may be involved in energy production from fatty acid derivatives (Landolt and Matile 1990, Pastori and del Río 1994). Furthermore, the activities of malate synthase and isositrate lyase, the key enzymes of the glyoxylate cycle, were found to be higher in peroxisomes isolated from senescent leaves than from non-senescent leaves (Pastori and del Río 1994).

1.2.1.3 Membrane degradation

Membrane deterioration during senescence is mainly due to phospholipid metabolism (Thompson et al. 1998). The lipolytic enzymes phospholipase D, phosphatidic acid phosphatase, lipolytic acyl hydrolase and lipoxygenase are found in senescing microsomal membranes. Phospholipase D is the key enzyme which most probably starts the breakdown of phospholipids, but lipoxygenase also has an important role during senescence since it can initiate lipid peroxidation and generate superoxide radicals (O_2^-) (Thompson et al. 1998). The accumulation of lipid metabolites in membranes causes membrane leakiness. During advanced senescence, membrane blebbing – the formation of lipid-protein particles – is impaired and thus more catabolites stay in the membranes.

1.2.1.4 Protein degradation

Besides nitrogen recycling, protein degradation affects receptor levels and signalling during senescence (Callis 1995). The ubiquitin-dependent protein degradation pathway works mainly in the cytosol and nucleus (Vierstra 1996). Until now, only a few genes involved in the ubiquitin-dependent protein degradation pathway (Buchanan-Wollaston 1997, Park et al. 1998) have been identified among senescence-associated genes (SAGs). Several other proteases, such as endoproteases, amino- and carboxypeptidases are abundant in senescing tissues (Huffaker 1990). Also, many cysteine-, aspartic- and metalloproteases have been identified among the senescence-upregulated genes (Solomon et al. 1999, Beers et al. 2000, Delorme et al. 2000). Protease activity is believed to be located mainly in vacuoles (Huffaker 1990), but there are proteases also in chloroplasts (Vierstra 1996, Adam 2002) as well as in peroxisomes (Distefano et al. 1999).

1.2.1.5 Modifications of nucleic acids

Generally, nuclear DNA remains constant and stays functional until the final stages of senescence (Feller and Fischer 1994), but some changes in DNA have been reported. For instance, an increased number of point modifications has been detected in senescing cork compared to young tissue in *Quercus suber* (cork-oak tree) (Pla et al. 2000). Also, increased

chromatin condensation and DNA fragmentation have been found during plant senescence (O'Brien et al. 1998, Yen and Yang 1998). The quantity of chloroplast DNA has been found to decrease when nitrogen deficiency induces leaf senescence in *Spinacia oleraceae* (spinach), while shading has no effect on the amount of chloroplast DNA (Scott and Possigham 1983). The level of total RNA decreases during senescence (Lohman et al. 1994, Masclaux et al. 2000) and chloroplast RNA declines at the same rate as the total RNA (Bate et al. 1991). This is connected to increased RNase activity during advanced senescence (Blank and McKeon 1991a, Blank and McKeon 1991b). Additionally, a bifunctional nuclease which is able to degrade both RNA and single-stranded DNA has been identified, and both the activity of this nuclease and the mRNA encoding it increase at the onset of leaf senescence (Pérez-Amador et al. 2000).

1.2.1.6 Reactive oxygen species and antioxidative system

Reactive oxygen species (ROS) are produced as part of normal cell metabolism, but during senescence the balance between production and elimination is disturbed (Thompson et al. 1987). ROS include the superoxide anion $O_2^{-}\cdot$, the perhydroxy radical $HO_2\cdot$, hydrogen peroxide H_2O_2, the hydroxyl radical $OH\cdot$, the alkoxy radical $RO\cdot$, the peroxy radical $ROO\cdot$, organic hydroperoxide ROOH, the singlet oxygen $^1O_2$, and the excited carbonyl RO* (Thompson et al. 1987). ROS can damage proteins, lipids, polysaccharides and nucleic acids causing deterioration of membranes, dysfunction of enzymes, and damage to DNA (Thompson et al. 1987). The production of some ROS, namely alkoxy radicals, peroxy radicals, and singlet oxygen, increases due to the increasing lipid peroxidation which occurs with advancing senescence (Thompson et al. 1987). Elevated levels of singlet oxygen induce other forms of ROS. Isolated peroxisomes from naturally senescing pea leaves have been shown to have elevated lipid peroxidation, and the levels of superoxide and hydrogen peroxide were also elevated (Pastori and del Río 1997). The levels of superoxide and hydrogen peroxide have also been found to be raised in mitochondria during dark-induced senescence of pea leaves (Jiménez et al. 1998).

The cellular antioxidative system consists of different enzymatic and non-enzymatic components (Noctor and Foyer 1998). The enzymes of the ascorbate-glutathione cycle are glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and ascorbate peroxidase (APX). In addition to these enzymes, superoxide dismutases (SOD), glutathione S-transferases (GST), catalases (CAT), glutathione peroxidase (GPX), and guaiacol-type peroxidases also participate in radical scavenging (Noctor and Foyer 1998). The major antioxidants besides ascorbate and glutathione are α-tocoferol, carotenoids, polyamines, and flavonoids. Different cell compartments have their own characteristic antioxidant systems consisting of different isoforms of antioxidant enzymes and antioxidants. The activities of antioxidative enzymes have been followed in *Cucumis sativus* (cucumber) cotyledons (Kanawaza et al. 2000) and in spinach leaves (Hodges and Forney 2000), both during natural senescence (Kanawaza et al. 2000) and after darkness- and ethylene-induced senescence (Hodges and Forney 2000). The results show that there are few similarities in antioxidative enzyme profiles between these different treatments. During natural senescence, the total activities of SOD, CAT, MDAR, DHAR and GR decrease while the activities of APX and guaiacol peroxidase increase (Kanawaza et al. 2000). Some organelle-specific studies have been done with mitochondria, peroxisomes and soluble fractions from senescing pea leaves (Pastori and del Río 1994, Pastori and del Río 1997, Jiménez et al. 1998). In these studies, dark-induced senescence caused a decrease in the activities of ascorbate-glutathione cycle enzymes and also SOD, with a simultaneous decrease

in ascorbate and glutathione content in mitochondria, whereas in peroxisomes the ascorbate and glutathione content and activities of GR and DHAR increased (Jiménez et al. 1998).

1.2.2 Senescence-associated genes

The terms *s*enescence-*a*ssociated *g*enes SAG (Gan and Amasino 1997), *sen*escence-associated genes SEN (Taylor 1989), *y*ellow-*l*eaf–*s*pecific genes YLS (Yoshida et al. 2001), *sen*escence-*u*pregulated genes SENU (Drake et al. 1996), and *se*nescence-*e*nhanced genes SEE (Smart et al. 1995) are used to describe genes that are expressed more actively during senescence. Several SAGs with putative or known function are suggested to be involved with changes in senescing cells (Table 1). These genes encode for example protease, ribonuclease, phospholipase D, catalase, chitinase, and ACC oxidase (Buchanan-Wollaston 1997). In addition, numerous genes are expressed during senescence whose functions are not yet known (Kleber-Janke and Krupinska 1997, Weaver et al. 1998, Huang,Y-J et al. 2001, Yoshida et al. 2001). Some regulatory genes that are expressed at the onset of senescence have been cloned. To date, the transcription factors isolated and found to be associated with senescence belong to the WRKY-family, which are zinc-finger-type proteins (Hinderhofer and Zentgraf 2001, Robatzek and Somssich 2001), and to the bZIP family, which are basic region leucine zipper proteins (Yang et al. 2001). Also, kinase genes have been characterized from senescing leaves: the senescence-associated receptor-like kinase from *Phaseolus vulgaris* (bean) (Hajouj et al. 2000) and a MAP kinase from *Zea mays* (maize) (Berbericch et al. 1999). Generally, the targets of these regulatory genes during senescence have not been identified, except in the case of WRKY6 in *Arabidopsis* (Robatzek and Somssich 2002). Putative target genes of *At*WRKY6 encoded protease, glutathione transferase, defence related genes, and one novel receptor-like protein kinase (Robatzek and Somssich 2002).

Two models for the timing of gene expression during leaf development have been suggested (Smart 1994, Buchanan-Wollaston 1997). Smart (1994) has described six classes and Buchanan-Wollaston (1997) has described ten classes of gene expression patterns based on the functioning of proteins in processes such as house-keeping, regulation, and mobilization. However, these models have not been exploited widely when new senescence-associated genes been have identified. The difficulty with these models is the comparison of different developmental stages between species and between experiments.

1.3 Regulation of leaf senescence

1.3.1 External factors

Plants are dependent on environmental conditions in which they grow, and changes in environmental factors have a remarkable effect on plant growth and the timing of senescence. Changes in growth conditions may have some regularity due to diurnal and seasonal rhythms, but may occur rapidly in an irregular way. Plants have to respond to all of these changes through fast metabolic adjustments (acclimation).

1.3.1.1 Light

The critical daylength for apical growth is the longest photoperiod under which growth cessation occurs (Håbjørg 1972). The critical daylength varies in Scandinavian tree ecotypes according to their provenance (Håbjørg 1978). In *Betula pubescens* (downy birch), this

variation can be up to 6-8 hours within Scandinavia (Håbjørg 1972). Besides the length of photoperiod, the quality and intensity of light also affect growth; increasing light intensity and also the proportion of red light have a positive effect on growth (Håbjørg 1972). On the contary, darkness is a widely used inducer of senescence for excised leaves (Pastori and del Rio 1991, Park et al. 1998, Weaver et al. 1998 Hodges and Forney 2000).

Shading at the whole-plant level can delay leaf senescence. When *Lolium temulentum* plants were transferred to a lower intensity of light at the onset of leaf senescence, leaf lifespan was prolonged and the rate of senescence was slower in shaded plants than in plants with the leaves ageing in full sunlight (Mae et al. 1993). Reduction in the amount of Rubisco and chlorophyll was slower in shaded plants than in plants with leaves ageing in full sunlight, and the rate of photosynthesis was higher in shaded leaves during advanced senescence (Mae et al. 1993). Studies of the effect of light intensity combined with different red/far-red (R/FR) ratios have revealed that the low R/FR ratio can be correlated with lower photosynthetic capacity and chlorophyll content in kidney bean leaves (Barreiro et al. 1992). R/FR ratio has been an important factor also in field conditions where the availability of light to lower leaves is often restricted because of neighbouring plants or self-shading. Rousseaux et al. (2000) have observed that basal leaf senescence of *Helianthus annuus* (sunflower) is affected both by decreasing R/FR ratio and decreasing photosynthetically active radiation. Furthermore, leaf senescence could be delayed if sunflower leaves were exposed to additional red light (Rousseaux et al. 2000).

1.3.1.2 Temperature

The length of the growing season also depends on temperature and the temperature sum (Koski and Selkäinaho 1982). The temperature sum is also involved in regulation of growth cessation in birch seedlings (Koski and Selkäinaho 1982). Interaction between temperature and photoperiod has been observed; a 5-10 ºC drop in day temperature caused earlier growth cessation but only when birches were grown below the critical daylength (Håbjørg 1972). Also, a drop in night temperature, below 9-10ºC, has caused apical growth cessation in *Salix pentandra* (bay willow) despite there being continuous light (Junttila 1980).

1.3.1.3 Other environmental factors

Leaf senescence can be initiated by limited availability of nutrients, i.e., nitrogen, potassium, phosphorus, magnesium, sodium and chlorine. Such deficiencies accelerate senescence in older leaves, enabling these mobile nutrients to be recycled into developing tissues (Thomas and Stoddart 1980, Marschner 1986). Slight water stress (Irigoyen et al. 1992) and the salinity of soil (Lutts et al. 1996, Vieira Santos et al. 2001) can also induce leaf senescence. Furthermore, changes in climate such as increasing ozone and carbon dioxide concentrations affect growth and the timing of leaf senescence. Exposure to ozone causes enhanced leaf senescence in deciduous tree species and is characterized by chlorosis, a decreased amount of Rubisco, and abscission of leaves (Pääkkönen et al. 1997a, Pell et al. 1999). When tobacco plants were grown in elevated carbon dioxide, the leaves started to senesce earlier than in ambient carbon dioxide concentration (Miller et al. 1997, Ludewig and Sonnewald 2000).

1.3.2 Internal factors

1.3.2.1 Ethylene

Ethylene production increases in leaves during the rapid chlorophyll loss (Aharoni and Lieberman 1979). Exogenously applied ethylene promotes leaf senescence, but this effect was found to be dependent on leaf age: mature leaves responded more strongly than leaves that were just fully expanded (Grbic and Bleecker 1995). Ethylene biosynthesis starts from methionine, which is converted in sequential reactions by *S*-adenosyl-L-methionine (SAM) synthetase, 1-aminocycloproprane-1-carboxylic acid (ACC) synthase, and ACC-oxidase to ethylene (Kende 1993). The rate-limiting step in ethylene biosynthesis is the formation of ACC by ACC synthase. When ethylene production decreased in ACC-oxidase antisense tobacco, leaf senescence was delayed up to two weeks but proceeded normally after this period (John et al. 1995). Ethylene seems to regulate the onset of senescence at a certain developmental stage of leaves; however, it is not necessary for the senescence process – either in *Arabidopsis* or tomato leaves (John et al. 1995).

1.3.2.2 Cytokinin

Exogenous application of several cytokinins such as kinetins (Richmond and Lang 1957), dihydrozeatin, zeatin, and benzylaminopurine (BA) have been shown to delay senescence in leaves of tobacco (Singh et al. 1992a) and in leaf discs of *Xanthium pennsylvanicum* (cocklebur) (Richmond and Lang 1957). Also, senescent tobacco leaves have re-greened when decapitated shoots were treated with BA (Zavalet-Mancera et al. 1999). In fact, the abundance of cytokinins changes throughout the development of leaves (Singh et al. 1992a, Van Staden et al. 1988). Singh et al (1992a) have reported that the sequential senescence of tobacco is connected to the cytokinin content of leaves, mainly zeatin. Generally, it is believed that cytokinins are synthesized mainly in roots and transported to leaves (Van Staden et al. 1988). However, cytokinins are also synthesized in young leaves of tobacco (Singh et al. 1992b) and the over-production of cytokinins in roots cannot prevent leaf senescence in tobacco (Faiss et al. 1997). Hence, local production of cytokinins in leaves might be a factor involved in the regulation of leaf senescence. There have been several attempts to delay leaf senescence by over-producing cytokinins with the *Agrobacterium tumefaciens iso*-pentenyl transferase (*ipt*) gene in transgenic tobacco lines (Smart et al. 1991, Gan and Amasino 1995, Faiss et al. 1997, McKenzie et al. 1998). Leaf senescence has, however, been successfully delayed only when the *ipt*-gene was under a copper-inducible promoter (McKenzie et al. 1998) or senescence-associated-gene (SAG) 12 promoter (Gan and Amasino 1995, Jordi et al. 2000).

1.3.2.3 Other plant hormones

The role and function of other plant hormones during leaf senescence has been studied less extensively. However, something is known about abscisic acid (ABA), jasmonates (JA), and gibberellins (GA) in relation to leaf senescence. The level of ABA increases before or during senescence, and decreases in the late stage of senescence (Noodén 1988). ABA promotes senescence, by being involved in chlorophyll breakdown and inhibition of chlorophyll biosynthesis (Noodén 1988). The levels of jasmonates have not been measured throughout leaf senescence but exogenous application of jasmonates has been shown to decrease chlorophyll content and depress the expression of photosynthetic genes (Creelman and Mullet 1997). Even though the roles of jasmonates and ABA during leaf senescence are not well characterized, both of them induce senescence in *Arabidopsis* leaves (Park et al. 1998, He et

al. 2001). In *Tropaeolum majus* (nasturdium) and *Alstroemeria hybrida* (alstroemeria), exogenous GA has been shown to delay leaf senescence (Beevers and Guernsey 1967, Kappers et al. 1998). When levels of endogenous gibberellins have been measured, GA_4 of the non-13-hydroxylated gibberellins decreased in concentration at the onset of dark-induced senescence before the loss of chlorophyll (Kappers et al. 1998). In addition to the above-mentioned hormones, at least salicylic acid, auxins, and brassinosteroids are involved in regulation of senescence (Noodén 1988, Clouse and Sasse 1998, Morris et al. 2000). Furthermore, interaction between different hormones is an essential part of regulation during plant development (Leopold and Noodén 1984). This hormonal control can be achieved by a balance or ratio between different hormones. Hormones can also have opposing effects on each other and their concentration may be altered by another hormone, or different hormones may function sequentially.

1.3.2.4 Metabolic signals

Metabolic signals such as the levels of carbohydrate and nitrogen, and also their ratio, have been found to affect leaf senescence (Paul and Driscoll 1997, Wingler et al. 1998). Carbon and nitrogen metabolites have been studied during leaf development by following changes from the highest leaves to the lowest leaves in tobacco (Masclaux et al. 2000). The sugar level was low in young leaves but increased in mature leaves that had started to senesce. The level of nitrate was low in young leaves and increased in old leaves, whereas the ammonium level was high in young leaves and decreased in mature leaves at the same time as sugar levels increased. This particular stage represents a transition point, when sink leaves turn to source leaves in primary assimilation and remobilization of nitrogen. Young leaves are sinks for free amino acids and carbohydrates because they have been growing and producing energy, while old leaves are sources of mineral nitrogen, amino acids and carbohydrates.

1.3.3 Signalling during senescence

The mechanisms for sensing changes in different light, temperature, water, and nutrient environments are currently under intense investigation. To date, some receptors and signalling substances have been discovered, but their exact function throughout the life span of the plant is not understood. Phytochromes are a class of photoreceptors which sense red and far-red light, and thus detect changes in daylight availability such as length of photoperiod and shading by neighbouring plants (Smith 2000). Phytochromes are encoded by a small family of genes where individual genes have their own specific roles in regulation of plant development (Smith 2000). Phytochromes can regulate gene expression and modify the balance of ions in the cell.

It has been proposed recently that nitrogen availability is mediated by cytokinin signalling (Sakakibara et al. 1998, Sakakibara et al. 2000). Nitrogen increases endogenous cytokinin levels (Singh et al. 1992c), and nitrogen deficiency causes a decrease in cytokinin levels (Horgan and Wareing 1980). Cytokinins regulate expression of several genes at the transcriptional and post-transcriptional levels (Schmülling et al. 1997), and cytokinins together with nitrogen regulate the expression of several photosynthesis and defence-related genes (Sakakibara et al. 2000).

There is some information available about metabolic changes that can be sensed by plants. Plants can sense levels of sucrose and hexose, and these sugars can act as signalling molecules (Smeekens 2000, Coruzzi and Zhou 2001). The level of glucose can be sensed via hexokinase which mediates the glucose signal for modifying gene expression of

photosynthetic genes *RbcS* and *Cab*, and also phospholipase D during senescence (Xiao et al. 2000).

Intracellular calcium concentration is often increased as a response to several kinds of abiotic stress (Bowler and Fluhr 2000) and during senescence (Huang et al. 1997). Calcium binds to calmodulin which mediates the calcium signal to calmodulin-binding proteins in cells. Recently, a gene encoding a calmodulin-binding protein was cloned from tobacco and it was shown to be expressed at high levels in senescing leaves (Yang and Poovaiah 2000). Generally speaking, calcium and other signalling, including their interactions during senescence, are still poorly understood.

1.4 Transgenic plants and mutants in senescence research

Chlorophyll loss is the visible sign of senescence. When stay–green varieties of different plants have been investigated, several mechanisms for maintaining greenness have been described (Thomas and Howart 2000). These mechanisms include (a) delaying the initiation of chlorophyll loss and photosynthetic capacity, (b) slowing the rate of decline, (c) maintaining high chlorophyll content even if photosynthesis declines. In addition, (d) sudden death by freezing can maintain the chlorophyll content, and (e) chlorophyll content may be higher than normal when the degradation is protracted. Stay-green varieties have been described for example in maize, *Sorghum bicolor* (sorghum), rice (Thomas and Smart 1993), and *Festuca pratensis* (fescue) (Thomas et al. 1992). As a consequence, they can have a higher yield and better resistance against drought and diseases (Thomas and Smart 1993). Some stay-green varieties have contained higher levels of cytokinins (Thomas and Smart 1993), have had a mutation in the nuclear *sid* gene (*s*enescence-*i*nduced-*d*eficiency) which prevents one step in chlorophyll degradation (Vicentini et al. 1995), or have inhibited chloroplast membrane degradation (Guiamét and Giannibelli 1994). When fescue with the *sid* mutation was studied further, it has shown to maintain a higher quantum efficiency of PSII in senescing leaves (Kingston-Smith et al. 1997). Part of this light energy might be used in carboxylation or oxygenation reactions of Rubisco, but the total activity of Rubisco also declined during leaf senescence of this mutant. The pool of reduced ascorbate was higher in the senescing leaves of *sid*-mutant than in wild-type fescue (Kingston-Smith et al. 1997). Also, the ratio of ascorbate to hydrogen peroxide was higher in the *sid*-mutant than in the wild-type, showing that excess light energy did not cause increased oxidative stress. Another study with the same *sid*-mutant of fescue has also shown that the decline in net photosynthesis is not connected to the chlorophyll content of leaves (Hauck et al. 1997). When growth and nitrogen content were analysed, higher nitrogen content was found in senescing leaves of the *sid*-mutant than in the wild-type due to limited remobilization (Hauck et al. 1997). These investigations have shown that high chlorophyll content in a stay-green mutant can affect the antioxidative status as well as the nitrogen metabolism of the plant.

Transgenic plants have been exploited in many studies of leaf senescence. As expected, cytokinin over-production (Gan and Amasino 1995, Jordi et al. 2000) and inhibition of ethylene production (Hamilton et al. 1990, John et al. 1995) delayed senescence. Cytokinin over-production in tobacco has also revealed that cytokinins interact with light and sugars during regulation of leaf senescence (Wingler et al. 1998). Light could partially block the promoting effect of sugars on leaf senescence. In turn, sugars could prevent the delaying effect of cytokinins on the reduction of photosynthetic proteins (Wingler et al. 1998). In the case of other transgenic plants, the enhancing or delaying effect on leaf senescence has been

unexpected. Overexpression of the *knotted1* gene (maize homeobox gene, transcription factor) under the senescence-specific cysteine protease (SAG12) promoter has caused delay in maize leaf senescence, probably due to increased cytokinin content (Ori et al. 1999). Also, in *RbcS* antisense tobacco plants that have reduced Rubisco levels leaf senescence phase was prolonged (Miller et al. 2000), while alterations in sugar sensing by overexpressing hexokinase has led to the hastening of senescence in tomato (Dai et al. 1999). Overexpression of phytochrome A has led to slowed chlorophyll degradation, causing an extension of lifetime and greater biomass production in *Solanum tuberosum* (potato) (Thiele et al. 1999). In *Populus tremula* x *tremuloides* (hybrid aspen), the critical daylength for elongation growth decreased from 15h in the wild-type to less than 6h in a transgenic line overexpressing phytocrome A, and the capacity for cold acclimation was lost (Olsen et al. 1997). Furthermore, when these plants were grown for 7 weeks under a short photoperiod (12 h) at 18°C and then transferred to low temperature (6°C) under an 11 h photoperiod, the wild-type leaves senesced and abscised within two weeks whereas PHYA-overexpressing lines retained most of their leaves.

Leaf senescence-associated genetic loci have been studied from stay-green sorghum in which seven quantative trait loci (QTLs) were associated with resistance to premature senescence under drought stress (Crasta et al. 1999). In *Arabidopsis* mutants, three genetic loci have caused delayed leaf senescence; one of these is allelic to *ein2*, a mutation causing ethylene insensitivity (Oh et al. 1997). Also, a *fireworks* mutant of *Arabidopsis* with early leaf senescence has been characterised (Nakamura et al. 2000). Generally, further investigations of these loci controlling senescence have not yet been reported. However, enhancer trap lines of *Arabidopsis* have been exploited to identify new senescence-associated genes and to study their expression also in response to several senescence-inducing factors (ABA, ethylene, dehydration, darkness, jasmonic acid, and brassinosteroids) (He et al. 2001). An enhancer trap line has one T-DNA insertion containing a marker gene and expression of the marker gene is detected when it is located near to a chromosomal gene. One-hundred and forty-seven lines expressing the marker gene during senescence have been selected for further studies. Different senescence-inducing factors have led to activation of specific groups of genes, but a large group of genes was not induced by any applied factors, showing that other factors are also involved in regulation of gene expression. Based on the patterns of gene expression after different treatments, a putative regulatory network between senescence-inducing factors has been suggested (He et al. 2001).

2 AIMS OF THIS STUDY

Leaf senescence is the final developmental stage of a leaf. Senescence is an important and interesting stage because it determines the active carbon assimilation period, and because nutrients are recycled from the leaf during senescence. In the last few years, leaf senescence has been investigated quite extensively in herbaceous plants such as *Arabidopsis*, tomato and tobacco. These annual herbaceous species have a short life-cycle which enables rapid production of the next generation. Also, transformation methods with high efficiency have been developed for these species, and genomic sequence information is available in the case of *Arabidopsis*. To date, little is known about the regulation of leaf senescence in deciduous tree species. This study focused on leaf senescence in silver birch during the first growing season, both in the greenhouse and in six-year-old trees growing in the field. Economically, silver birch is the most important hardwood tree species in Finland, and is widely distributed in Eurasia.

2.1.1 Characterization of leaf senescence in silver birch (I, III, additional data)

The aim was to study the timing of photosynthesis and senescence during leaf ontogeny in silver birch. Silver birch follows an indeterminate growth pattern, enabling growth as long as the growing conditions are favorable. The growth of silver birch leaves is exceptional during the first growing season, when all leaves are formed gradually in the developing shoot. During the following growing seasons, the short shoot leaves burst simultaneously from buds in the spring. They are maintained throughout the growing season while the long shoot leaves gradually develop into the growing long shoot. Due to the different growth patterns of silver birch leaves, leaf ontogeny was studied both during the first growing season with silver birch seedlings under greenhouse conditions (I) and also with six-year-old silver birch clones K1659 and V5952 grown in the field (additional data). In addition, leaf senescence was studied in a late-senescing silver birch line (R3.1) which had been produced by insertion mutation in this study (III). Leaves of line R3.1 were compared to wild-type clone R leaves.

To determine different developmental stages during leaf ontogeny, photosynthesis was followed by measuring the efficiency of PSII, by biochemical analyses of chlorophyll content, and also by determining the amount and activity of Rubisco. The aim was to study the timing of gene expression during leaf development and senescence with the genes presented in Table 2. Most of these genes are oxidative-stress related genes and have been isolated and studied under ozone stress (Tuomainen et al. 1996, Kiiskinen et al. 1997, and Korhonen, Tuominen, Vahala, and Kangasjärvi, unpublished), pathogen invasion (Pellinen et al. 2002) and heavy metal treatment (Utriainen et al. 1998). Since ROS are involved in cell metabolism during leaf senescence (Thompson et al. 1987, Pastori and del Río 1997, Jiménez et al. 1998), the aim was to identify which oxidative-stress related genes are also senescence-associated genes in silver birch. The genes studied also included photosynthesis-related genes, in order to compare biochemical analyses of photosynthetic activity and cysteine proteases as potential molecular markers of leaf senescence. Some genes such as those encoding allene oxide synthase and dehydrin have been isolated when cold acclimation of silver birch has been studied (Aalto, Puhakainen and Palva, unpublished).

Table 2. List of silver birch genes investigated with regard to role in leaf senescence in the present study, and reports from the literature on the expression of genes in other plant species during senescence. ↑ = mRNA level increased during senescence; ↓ = mRNA level decreased during senescence; NA = gene expression not analysed during leaf senescence.

Gene	Plant species and reference
Photosynthesis	
RbcS, small subunit of Rubisco	↓ kidney bean (Bate et al. 1991), soybean (*Glysine max*) (Jiang et al.1993)
Cab, chlorophyll a/b binding protein	↓ kidney bean (Bate et al. 1991)
Ca, carbonic anhydrase	NA
Energy production	
Mpt, mitochondrial phosphate translocator	NA
G6PD, glucose-6-phosphate dehydrogenase	NA
Adh, alcohol dehydrogenase	NA
Cell maintenance and development	
Lox, lipoxygenase	NA
Lap, leucine aminopeptidase	NA
Cyp, cysteine protease	↑ tomato (Drake 1996), tobacco (Ueda et al.2000)
Atub, alpha-tubulin	NA
Dad, defender against apoptotic cell death	↑ pea (*Pisum sativum*) (Orzaéz and Granell 1997)
Dhn, dehydrin	NA
Antioxidants	
Apx, ascorbate peroxidase	↑ tomato (Gadea et al. 1999)
Gpx, glutathione peroxidase	NA
Gr, glutathione reductase	NA
Gst, glutathione-S-transferase	↑ *Dianthus caryophyllus* (carnation) (Itzhaki et al. 1994)
Cu/Zn sod, Cu, Zn superoxide dismutase	↓ tobacco (Kurepa et al. 1997)
Cat, catalase	↑ *cat1*, ↓ *cat2*, *cat3 Cucurbita* (pumpkin) (Esaka et al. 1997)
Defence genes	
PR1, pathogenesis-related protein 1	↑ *Brassica napus* PR-1a-like and class IV chitinase (Hanfrey et al. 1996)
PR3a, acidic endochitinase	
Ypr10, pathogenesis-related protein 10	↑ *Glysine max* soybean (Crowell et al. 1992)
Wrky, DNA binding protein wrky	↑ *Arabidopsis thaliana* WRKY53 (Hinderhofer and Zentgraf 2001), WRKY6 (Robatzek and Somssich 2001)
Hormone biosynthesis and perception	
Bp-ACO1, ACC oxidase	↑ tomato, *Nicotiana plumbaginifolia* (Blume and Grierson et al. 1997)
Bp-ACS, ACC synthase	↑ carnation (Jones and Woodson 1999)
Bp-ETR, ethylene receptor	no change or ↓ tomato (Lashbrook et al 1998)
Aos, allene oxide synthase	↑ *Arabidopsis thaliana* (Kubigsteltig et al. 1999)

Gene	Plant species and reference
Phenylpropanoid biosynthesis	
DS3, 3-deoxy-D-arabinoheptulosonate-7-phosphate	NA
Pal, phenylalanine ammonium lyase	NA
Chs, chalcone synthase	NA

2.1.2 Genetic transformation of silver birch and experiments with transgenic silver birch lines (II, IV)

The use of transgenic trees has been restricted mainly to the species of genus *Populus* due to effective genetic transformation methods (Klopfenstein et al. 1997, Leplé et al. 2000). There is a need to develop transformation methods for silver birch, both for breeding purposes and for research on basic mechanisms of genetic regulation of growth and development. Silver birch has a diploid genome and therefore genetic studies are easier than with hybrid poplars. In the present study, the aim was to produce transgenic silver birch lines via antisense-*RbcS* suppression for further research on C/N metabolism (II). Also, a sense-*RbcS* construct was used in transformation work to possibly cause co-suppression but over-production of *RbcS* in chloroplasts was prevented by removing transit peptide from the *RbcS*-construct (II). The long-term aim was to reduce the amount of Rubisco, the key enzyme of carbon metabolism, in order to investigate how photosynthesis and growth are affected in silver birch lines containing less Rubisco, both under optimal growth conditions and under elevated carbon dioxide concentration. These silver birch lines might be used to answer questions such as whether silver birch with a reduced amount of Rubisco could photosynthesize and grow under elevated carbon dioxide as well as wild-type silver birch under ambient carbon dioxide concentration. Antisense *RbcS*-transformed tobacco (Masle et al. 1993) and rice (Makino et al. 1997) plants with decreased Rubisco content have been exploited when changes in photosynthesis and efficiency of nitrogen use have been investigated under elevated carbon dioxide concentration. The results have partly been species-specific, and therefore it would be interesting to study how reduction of Rubisco would affect carbon and nitrogen metabolism and their interaction in trees. The maintenance of interesting and important transgenic silver birch lines as *in vitro* cultures is laborious and prolonged *in vitro* cultivation is not preferable due to the risk of contamination and somaclonal variation (Kaeppler et al. 2000). Since cryopreservation has proved to be a successful method for preservation of *in vivo* cold-hardened buds (Ryynänen 1996, Ryynänen 1999), the aim was to apply cryopreservation to the long-term maintenance of transgenic silver birch lines (IV).

3 MATERIALS AND METHODS

3.1 Plant materials, growing conditions, and experimental design

3.1.1 Senescence studies

3.1.1.1 Seedlings in greenhouse (I)

The silver birch seedlings were grown at Suonenjoki Research Station (62°40'N, 27°00'E) from local seed. The seedlings were grown in a greenhouse under a photoperiod of 18 hours of light and 6 hours of darkness. The seedlings were grown in pots containing fertilized peat and optimal nutrition was maintained with fertilization. Leaves at four different positions representing developmental stages from young to mature were marked in the silver birch seedlings two months after sowing. These marked leaves and also new leaves developing above were collected for analysis during the following seven weeks (Fig. 1A in I). Development of leaf area was recorded to determine when the leaves were fully expanded (Fig. 1B in I). The amount of Rubisco, mRNA levels of *RbcS*, and the amount of total soluble protein and chlorophyll were determined in order to follow changes in the activity of photosynthesis and leaf development during leaf ontogeny. Furthermore, the expression of ACC-oxidase (*Bp-ACO*), pathogenesis-related protein 10 (*Ypr10*), mitochondrial phosphate translocator (*Mpt*), and leucine aminopeptidase (*Lap*) was studied in order to determine their role in leaf senescence.

3.1.1.2 Young trees in field experiment (additional data)

The experimental field located at Suonenjoki Research Station contained 15 silver birch clones. Detailed descriptions of the field experiment can be found in Mutikainen et al. (2000). Short and long shoot leaf ontogeny was studied in two clones, an ozone-sensitive clone (K1659) and an ozone-tolerant clone (V5952) (Pääkkönen et al. 1997b) throughout the growing season of 1997. Twenty trees of each clone growing in different parts of the field were selected for the study. The 6-year-old trees were growing in sandy soil and received additional fertilization of 22 kg N /ha $^{-1}$ (N:P:K 18:5:10, Typpirikas Y-lannos, Kemira Ltd., Helsinki, Finland) during the growing season of 1997. Weather conditions, daily mean, minimum and maximum temperatures, temperature sum, and rainfall during the growing season of 1997 are shown in Figure 1.

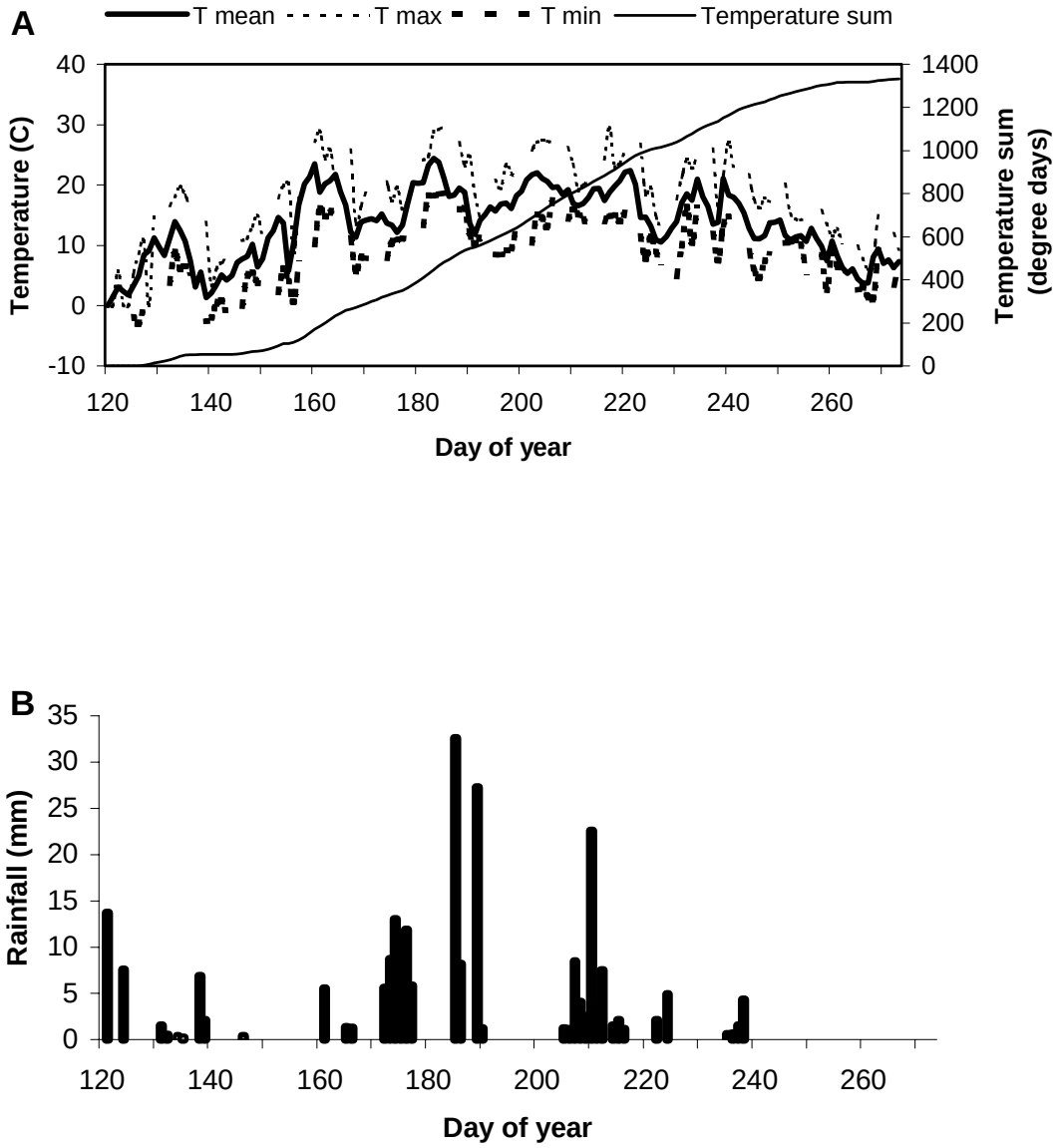

Figure 1. A. Maximum, minimum, and mean air temperature, and the effective temperature sum in Suonenjoki (degree days above the threshold of 5°C). B. Daily rainfall (mm) at the site during the growing season of 1997. May 1 is day (DOY) 121.

The short shoot leaves (leaf 0) that burst simultaneously in spring from buds formed during the previous growing season were collected on June 2, 12, 27, and August 21 (day of year, DOY 153, 163, 178, and 233) during the growing season of 1997. The long shoot leaves that first developed into the growing long shoot (leaf 1) were collected on June 27, July 24, and August 29 (DOY 178, 205, and 241). The leaves that developed next into the long shoot were marked as leaf 2 and were collected on July 28 and August 29 (DOY 209 and 241). On the last day of sampling, October 2 (DOY 275), the long shoot leaves collected were from the third to fifth in order of development and they were marked as leaf 3 (Figs. 2, 3, 4, 5). The leaves sampled were located in the lower half of the crown, at a height of 1.8 m or below, and

they were collected randomly from all branches during the early afternoon. Leaves from five replicate trees were pooled together to make a replicate sample, and hence a total of four replicates from 20 trees was obtained from each clone. Two sets of leaves were collected, the first set being for determinations of leaf area, and fresh and dry weight, and the second for analysis of the amount of Rubisco, the initial activity and the total activity of Rubisco, the amount of chlorophyll and total soluble protein, and for analysis of gene expression.

Photosynthesis in the short and long shoot leaves was studied by following changes in light reactions by chlorophyll fluorescence measurements. The maximal capacity of PSII after a 15 min dark-adaptation (Fv/Fm) was measured between 10 a.m. and 16 p.m. from short shoot leaves on June 3, 12, 16, 18, 30, July 18, and August 22 (DOY 154, 163, 167, 169, 181, 199, and 234, respectively). On July 2 and 21 (DOY 183 and 202), Fv/Fm was measured from the long shoot leaves that developed first. Fv/Fm was then measured from the second long shoot leaves on July 15 (DOY 196). The actual efficiency of PSII in light (Fv'/Fm') in the above leaves was recorded from June 30 onwards. Gas exchange was measured from the first and the second long shoot leaves on August 5-6 (DOY 217-218).

3.1.1.3 Late-senescing silver birch mutant (III)

The late-senescing R3.1 line and the wild-type clone R plantlets were planted in fertilized peat and grown in greenhouse conditions under a photoperiod of 16 hours of light and 8 hours of darkness. After a five-week precultivation period, young developing leaves were marked as lifespan leaves which would be followed for the ensuing seven weeks in order to compare developmental changes in wild-type clone R with those in the late-senescing dwarf line R3.1. In addition, the youngest fully expanded leaves were collected on each sampling day to represent the most actively photosynthesizing leaf. This was carried out in clone R, but in line R3.1 leaf development was so slow that new fully expanded leaves were collected only on the last two sampling days. Changes in leaf development were followed by measuring leaf-area, maximal and actual efficiency of PSII, activity and amount of Rubisco, chlorophyll, and soluble protein. Gas exchange was measured to explore possible differences in net photosynthesis in the youngest fully developed leaves. Samples were also taken for electron microscopy to detect possible structural differences between these lines. Leaf senescence-related gene expression was studied by reverse northern and RNA dot-blot hybridization analysis of a number of genes, listed in Table 2.

3.1.2 Genetic transformation studies

3.1.2.1 Biolistic transformation (II)

The *in vitro* silver birch clones used for developing the biolistic transformation method were derived from elite genotypes (II). The clones used for genetic transformation experiments were R, V5952, E5389, A, E5396, 98, E5382, E5398, E5387, E5201, and E1987 which were maintained as *in vitro* cultures at Punkaharju Research Station. Leaf and shoot pieces of the *in vitro* clones were used as explant material after different precultivation periods on different tissue culture media (II). Regenerated lines from the R and E5396 clones were further cultivated under greenhouse conditions to obtain leaf material for molecular analysis in order to confirm transgene integration (II, IV).

3.1.2.2 Cryopreservation studies (IV)

The transgenic lines used in this study were transformed with sense-*RbcS* pRT99 or pRT99gus representing clones R and E5396. The one- and two-year-old transgenic lines which had been grown in the greenhouse were transferred into dark, cold storage at +2°C for two months, for cold hardening treatment. Stem and branch buds were collected in the middle of March and cryopreserved. After cryostorage, buds were thawed and cultivated on woody plant medium for four weeks while regeneration was followed. Regenerated *in vitro* plantlets were transferred onto Murashige-Skoog's medium for rooting. At the end of May, plantlets were potted in peat-soil mixture and transferred to greenhouse conditions. After a few weeks of growth in the greenhouse, leaves from cryopreserved and non-cryopreserved lines were collected for analysis of neomycin phosphotransferase (*nptII*) gene stability and expression.

Table 3. Description of the experiments

Article	Sampling period	Experimental material	Location, photoperiod	Scope of the study
I	7 weeks	Eight week-old seedlings	Greenhouse, 18 h	To study leaf ontogeny in silver birch seedlings during their first growing season
II		*In vitro* clones R, V5952, E5389	Growth room, 16 h	Stable transformation of silver birch
	One sampling	Lines R3.1 and R7.2	Greenhouse, Natural	
III	10 weeks	Seven week-old plants from clone R and line R3.1	Greenhouse, 16 h	To study leaf ontogeny in clone R and line R3.1 during their first growing season
IV	One sampling	1- and 2-year-old plants from lines R7.2, R3.2, E1, E5 and wild-type R and E	Greenhouse, natural	Cryopreservation of transgenic silver birch lines
Additional data	18 weeks	6-year-old birches from clones K1659 and V5952	Field, Natural	To study leaf ontogeny of short and long shoot in young trees

3.2 Methods used in the studies

3.2.1 Photosynthesis measurements

Chlorophyll fluorescence of leaves was measured at growth temperature using a portable, pulse-amplitude-modulated fluorometer (MINI-PAM, Heinz Walz GmbH, Effeltrich, Germany) (III, additional data). After 15 min of dark adaptation, the minimal fluorescence level (F_0) was determined using a pulsed low-intensity modulated measuring light (<0.1 μmol m^{-2} s^{-1}). A saturating pulse of about 9000 μmol m^{-2} s^{-1} was used to obtain the maximal fluorescence level (F_m). The maximal quantum yield of PSII photochemistry in a dark-adapted state (F_v/F_m) was calculated as (F_m - F_0)/F_m (Genty et al. 1989). Thereafter, the leaves were exposed to actinic light (External Halogen Lamp 2050-H, <700 nm, Halogen Masterline Dichroic, 8° beam divergence angle, Philips) that varied between 458 and 850 μmol m^{-2} s^{-1} (additional data), and between 360 and 475 μmol m^{-2} s^{-1} (III) for at least 2 min, after which steady-state fluorescence (F_s) was recorded, and a saturating pulse was applied to determine the maximal fluorescence at steady-state (F_m'). The quantum yield of PSII photochemistry at light-adapted steady-state (F_v'/F_m') was calculated as ($F_m' - F_s$)/F_m' (Genty et al. 1989).

Gas exchange was measured with a closed-system infrared gas analyser (LI-6200, LiCor Inc., Lincoln, Nebraska, USA) from the leaves of field-grown trees (Table 4). The photosynthetic rates of leaves were measured at a CO_2 concentration of 330 to 340 ppm in natural light (120-1930 μmol $m^{-2}s^{-1}$). Ambient relative humidity (RH) varied between 20% and 56%, and RH in the leaf chamber was adjusted to be close to this. Leaf temperature was between 27°C and 38°C during measurements. In the greenhouse study (III), gas exhange was measured using an open-system gas analyser (LI-6400, LI-COR Inc.) with a standard leaf chamber equipped with 6400-02B LED Light Source (LI-6400, LI-COR Inc.). Gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981).

Biochemical analyses for measuring the amount of Rubisco (I, III, additional data), the initial activity of Rubisco (additional data) and total activity of Rubisco (III, additional data), amount of chlorophyll (I, III, additional data), and total soluble protein (I, III, additional data) were performed from leaf samples frozen in liquid nitrogen after homogenization in extraction buffer (III). Aliquots of the crude extract were dissolved in 80% buffered acetone and chlorophyll was determined by the method of Porra et al. (1989). After 2 min centrifugation, the initial and total activity of Rubisco was determined as incorporation of ^{14}C into acid-stable products (Laitinen et al. 2000). The amount of Rubisco protein was determined by PAGE (Ruuska et al. 1994), and the soluble protein content was measured by the method of Bradford (1976).

For electron microscopy (III), leaf samples were kept in the fixative at +4°C for about 22 h. Further stages of fixation were carried out as described by Soikkeli (1980). Leaf sections were stained with uranyl acetate and lead citrate. The palisade and spongy cells were photographed with a transmission electron microscope (JEOL JEM-1200 EX) using a digital system.

3.2.2 Gene expression analysis

The expression of photosynthesis and senescence-related genes was studied by RNA dot-blot hybridization analysis (I, III, additional data), northern hydridization analysis (III, IV), and reverse northern hybridization analysis (III, additional data). Total RNA was extracted according to Chang (1993) and mRNA was isolated by poly(A)$^+$RNA purification (Oligotex ^{TM} mRNA midi kit, Qiagen GmbH, Germany).

In the leaves of field-grown silver birch clones (additional data), both reverse northern and RNA dot-blot hybridization analysis were performed as in (III). The actively photosynthesizing leaf 0 from the short shoot on DOY 151 and the clearly senescing leaf 3 from the long shoot on DOY 248 from clone V5952 were compared by reverse northern hybridization analysis (Table 2). As a constitutively expressed control gene during leaf development, a translation initiator factor (from silver birch est-sequencing project, Tapio Palva) was used because its mRNA levels varied less than twofold in northern hybridization analysis. RNA dot-blot analysis with selected genes was performed with 5 µg of total RNA to confirm the results obtained from reverse northern hybridization analysis.

3.2.3 Genetic transformation

The vectors used for developing the biolistic transformation method, pRT99gus (Töpfer et al. 1988), pBI221.1 (Jefferson 1987), and pBI426 (Datla et al. 1991, Charest et al. 1993), were received as gifts; or, in the case of pRT99 (II), constructed by routine cloning methods. In order to avoid over-expression in the chloroplast, sense-*RbcS* and antisense-*RbcS* constructs did not contain transit peptide of *RbcS*. The plasmids were precipitated on gold particles and a biolistic PDS®-1000/He device was used for bombardment (II). The transformation efficiency was determined at the transient GUS (ß-glucuronidase) expression level when different *in vitro* callus cultures originating from elite silver birch genotypes, vectors, or length of precultivation time before bombardment were compared (II).

To produce transgenic silver birch lines, antisense-*RbcS* pRT99, sense-*RbcS* pRT99, and pRT99gus vectors were used to the same extent in biolistic transformation experiments. After transformation, different tissue culture media were tested for regeneration (II). DNA was isolated from the greenhouse-cultivated silver birch lines (II) and was used for Southern hydridization analysis to study integration of transgenes (II).

3.2.4 Cryopreservation

The cryopreservation protocol was based on the so-called slow-cooling method. Cryopreserved buds were thawed and placed on tissue culture media for regeneration (IV). Two and four weeks after thawing, the proportion of regenerated buds was calculated. The stability and expression of inserted *nptII*-genes were investigated from leaves of regenerated plants by Southern and northern hybridization analysis.

4 RESULTS

4.1 Leaf senescence in silver birch

4.1.1 Seedlings in greenhouse (I)

Photosynthesis and senescence-related changes were followed throughout leaf development in different leaf positions of silver birch seedlings. Photosynthesis increased until leaves were fully expanded, which is based on trends observed in the amount of Rubisco protein and mRNA level of *RbcS* (Figs. 2C and 3A in I). Thereafter, a steady decrease could be observed when leaves became self-shaded and shaded by neighbouring seedlings. Simultaneously with the decline in the amount of Rubisco, the amount of soluble proteins also started to decrease in seedlings (Figs. 2B and 2C in I), but this seemed to proceed more slowly than the loss of Rubisco protein (Fig. 2C in I). The chlorophyll content of leaves decreased later than the soluble protein content (Figs. 2A and 2B in I). Senescence-related changes in gene expression in silver birch seedlings were investigated with *Lap*, *BP-ACO1*, *Mpt1*, and *Ypr10* probes (Fig. 4 in I). Their mRNA levels increased in the older leaves but not simultaneously, each gene having its own typical expression pattern (Fig. 4 in I).

4.1.2 Young trees in field experiment (additional data)

Development of short shoot leaves and long shoot leaves was followed in six-year-old silver birches representing clones K1659 and V5952. Short shoot leaves expanded rapidly at the beginning of June (DOY 126-136) (Fig. 2), while expansion of long shoot leaves was much slower from the end of June to August (DOY 178-241). In both the short and long shoot leaves, photosynthetic capacity increased along with leaf expansion (Fig. 2) when the maximal capacity of PSII, Fv/Fm (Fig. 3A), amount and activity of Rubisco (Figs. 4 and 5A) were used as indicators. The mRNA level of *RbcS* increased in short shoot leaves when they expanded, but expression of *RbcS* was already high in young long shoot leaves (leaf 1) (Fig. 6A).

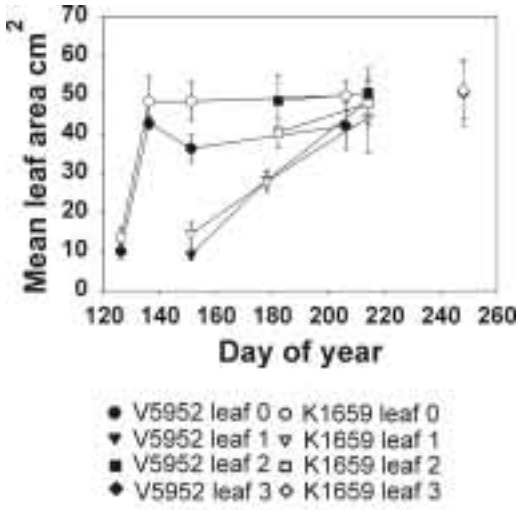

Figure 2. Leaf area development in clones K1659 and V5952 during the growing season of 1997. Leaf 0 was a short shoot leaf and leaves 1, 2, and 3 were from the long shoot. The data show the mean area (± SE) of four replicate samples, each consisting of five leaves.

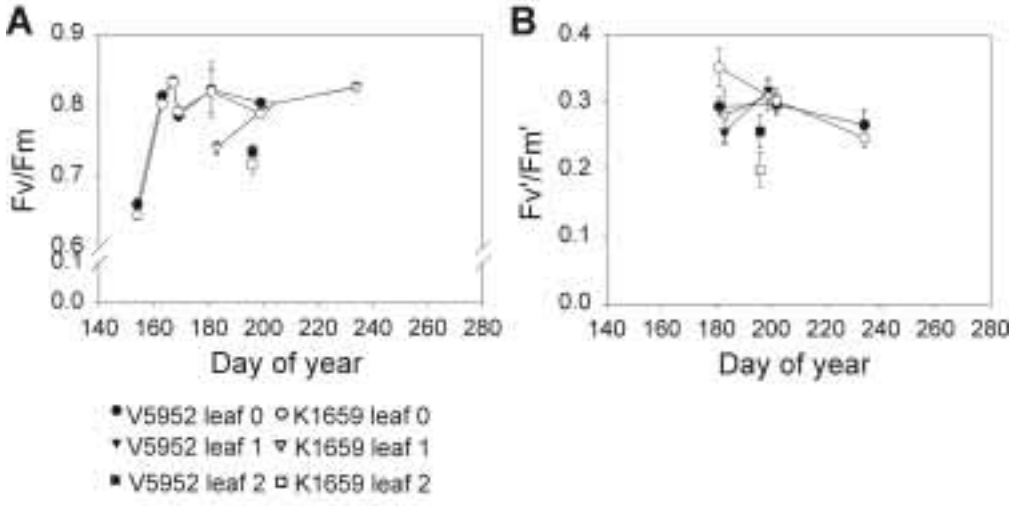

Figure 3. The maximal efficiency of PSII in dark-adapted state (A) and the actual efficiency of PSII in light (B) measured from short (leaf 0) and long shoot leaves (leaves 1, 2) of clones K1659 and V5952. The data are the means (± SE) of 20 measurements. After 15 min of dark adaptation, the minimal fluorescence level (F_0) was determined using a pulsed low-intensity modulated measuring light. A saturating pulse of about 9000 μmol m^{-2} s^{-1} was used to obtain the maximal fluorescence level (F_m). The maximal quantum yield of PSII photochemistry in a dark-adapted state (F_v/F_m) was calculated as (F_m - F_0)/F_m. Thereafter, the leaves were illuminated at 458-850 μmol m^{-2} s^{-1} for a minimum of 2 min, after which steady-state fluorescence (F_s) was recorded, and a saturating pulse was applied to determine the maximal fluorescence at steady-state (F_m').

After short shoot leaves had reached their full size (Fig. 2), the amount of Rubisco (Fig. 5), the total and initial activity of Rubisco (Fig. 4), the *RbcS* mRNA level (Fig. 6A), and soluble

protein content (Fig. 5C) decreased slowly during the weeks that followed. Chlorophyll content did not decrease in the short shoot leaves sampled during the experiment (Fig. 5B).

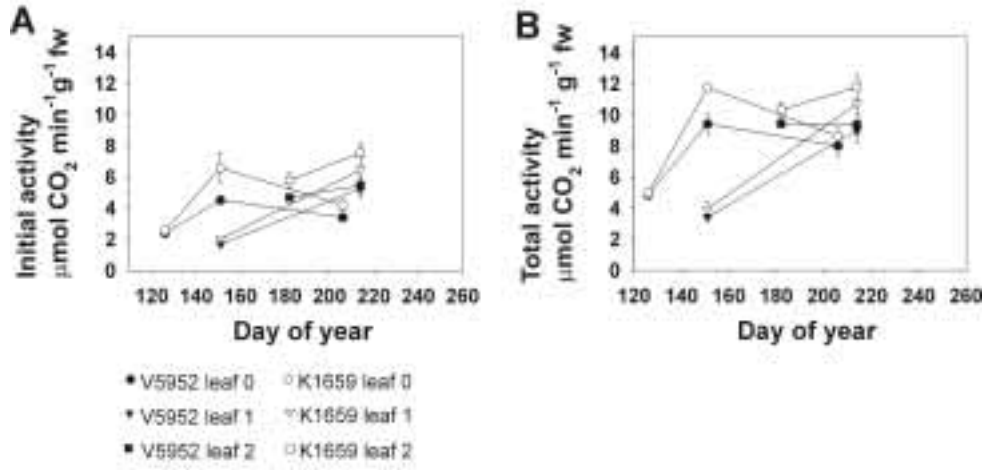

Figure 4. The initial activity (A) and total activity (B) of Rubisco in short (leaf 0) and long shoot leaves (leaf 1, leaf 2) of clones K1659 and V5952. The data are the means of four replicates ± SE.

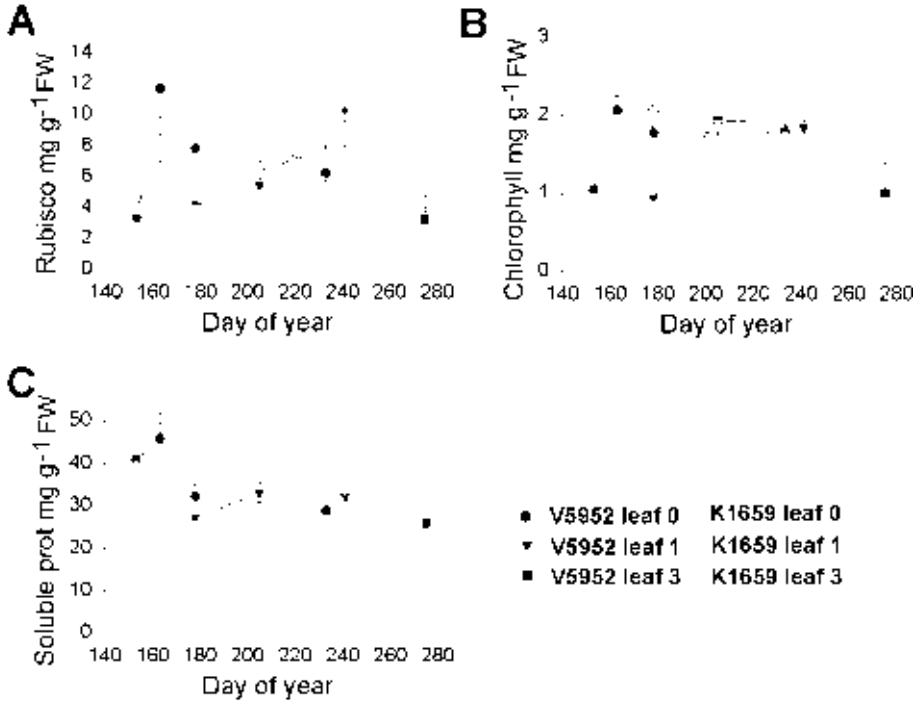

Figure 5. The amounts of Rubisco (A), chlorophyll (B), and soluble proteins (C) in short (leaf 0) and long shoot leaves (leaf 1, leaf 3) of clones K1659 and V5952. The data are the means of four replicates ± SE.

Table 4. Gas exchange on DOY 217-218 in long shoot leaves (leaf 1, leaf 2) of clones K1659 and V5952 measured with a gas analyser (LI-6200, LiCor Inc.) During measurements, CO_2 concentration was between 330 and 340 ppm, natural light level was 120-1930 μmol $m^{-2}s^{-1}$, RH varied between 20% and 56%, and leaf temperature was between 27°C and 38°C. The data are the means of 7-8 leaves ± SE.

			K1659	
	V5952 leaf 1	leaf 2	leaf 1	leaf 2
Net phosynthesis, μmol $m^{-2}s^{-1}$	15,6 ± 0,9	15,0 ± 2,0	16,3 ± 1,3	21,7 ± 1,3
Stomatal conductance, mol H_2O $m^{-2}s^{-1}$	0,2 ± 0,0	0,2 ± 0,0	0,3 ± 0,0	0,4 ± 0,1
Intercellular CO_2 concentration, μmol mol^{-1}	158,9 ± 25,9	187,2 ± 6,0	196,7 ± 21,5	202,4 ± 6,2
Transpiration, mol H_2O $m^{-2}s^{-1}$	4,8 ± 0,3	6,4 ± 0,7	5,3 ± 0,1	10,6 ± 1,0
Intercellular CO_2 / atmospheric CO_2	0,5 ± 0,1	0,6 ± 0,0	0,6 ± 0,1	0,6 ± 0,2

On DOY 241, when the first long shoot leaves (leaf 1) were fully expanded, the initial and total activity of Rubisco (Fig. 4B), amount of Rubisco (Fig. 5A), mRNA level of *RbcS* (Fig. 6A), and chlorophyll content (Fig. 5B) were similar to corresponding values for the young, fully expanded short shoot leaves (leaf 0 on DOY 163). Photosynthesis decreased clearly in the long shoot leaves (leaf 3) on DOY 275, based on the amount of Rubisco and chlorophyll (Figs. 5A and 5B), and also on the mRNA level of *RbcS* (Fig. 6A). There was a slight decrease in soluble protein content (Fig. 5C) on DOY 275.

Differences between clones were found in the initial and total activity of Rubisco (Fig. 5), in gas exhange (Table 4), and in gene expression (Fig. 6). Clone K1659 had slightly higher initial and total activity of Rubisco (Fig. 5). Net photosynthesis and also the intercellular CO_2 were higher in long shoot leaves of clone K1659 than in corresponding leaves of clone V5952 (Table 4). This was partly due to higher stomatal conductance found in K1659. Differences in gene expression were detected in the mRNA levels of *RbcS* and *Pr1*, the mRNA levels of *RbcS* and *Pr1* being higher in clone K1659 than in clone V5952 on some sampling days (see Figs. 6A and 6E).

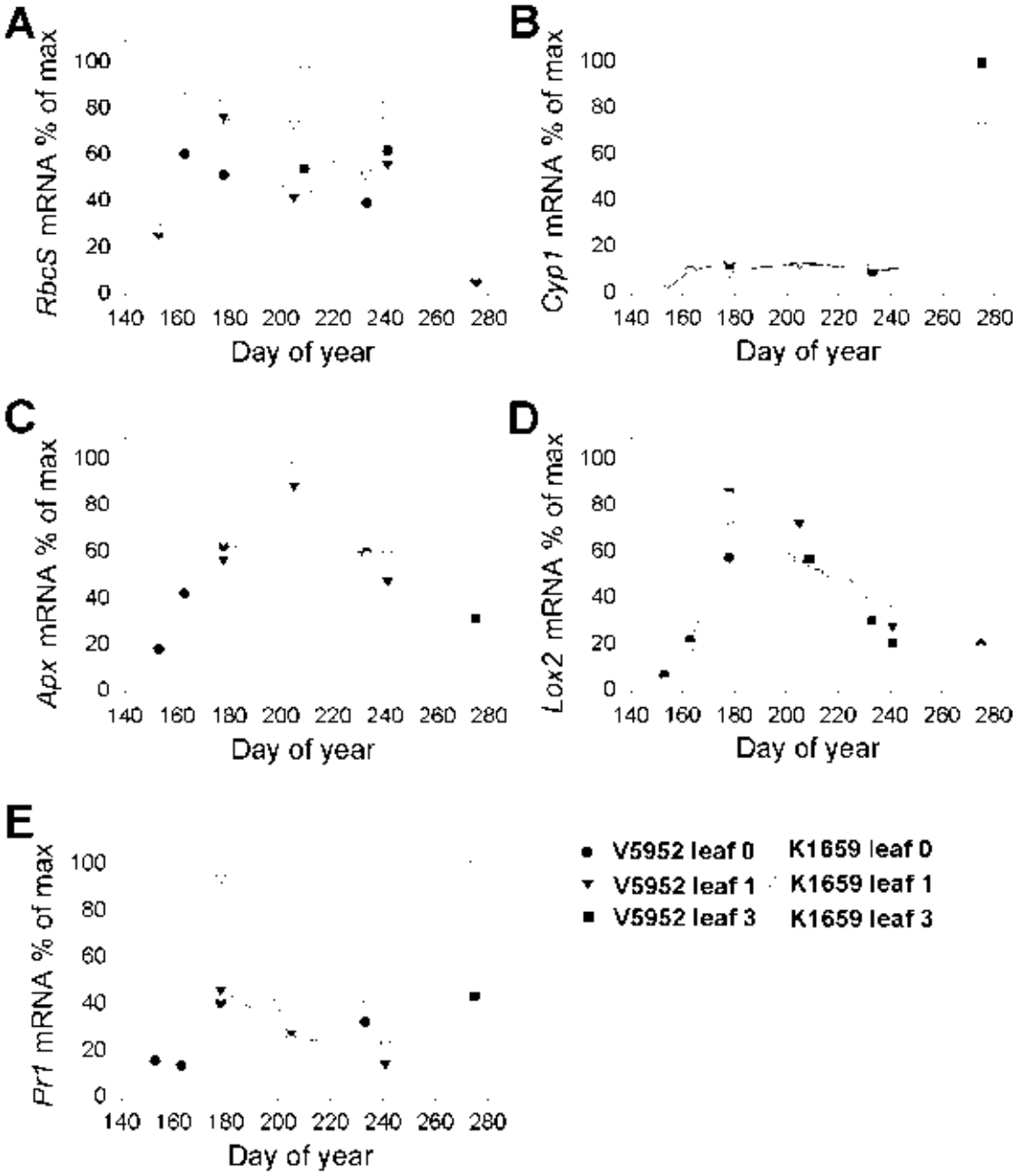

Figure 6. The mRNA levels of *RbcS* (A), *Cyp1* (B), *Apx* (C), *Lox2* (D), and *Pr1* (E) relative to 18S were quantified by RNA dot-blot hybridization analysis from short shoot (leaf 0) and from long shoot leaves (leaf 1, leaf 3) of clones K1659 and V5952.

The expression of silver birch genes listed in Table 5 was studied during leaf senescence in clone V5952. Almost half of the genes studied showed no change in gene expression when the actively photosynthesizing short shoot leaf from DOY 178 and clearly senescing long shoot leaf from DOY 275 were compared (Table 5). Transcript levels of photosynthesis-related genes decreased clearly during leaf senescence (Table 5). RNA dot-blot hybridization analysis with *RbcS* (Fig. 6A) from short and long shoot leaves of clones K1659 and V5952 showed that the mRNA levels of *RbcS* were higher in clone K1659 than in clone V5952, both in short and long shoot leaves. A marked decrease in *RbcS* mRNA was visible at the last sampling, DOY 275 (Fig. 6A). During leaf senescence, the following studied genes were

induced in clone V5952 (Table 5): *G6PDH* and *Adh* (energy metabolism), and *Lap*, *Cyp1*, *Cyp2*, and *Dhn* (cell maintenance and development), *Gr* (antioxidants), *Bp-ACO1* and *Aos* (hormone biosynthesis and perception), and *Pal* (phenylpropanoid biosynthesis). RNA dot-blot hybridization analysis revealed some changes during leaf development and some differences between clones that were not detected with reverse northern hybridization analysis. Firstly, the mRNA levels of *Apx* were high in fully developed short and long shoot leaves (Fig. 6C). Secondly, the mRNA level of defence-related gene, *Pr1*, increased in long shoot leaves of clone K1659 in the middle and at the end of the growing season (Fig. 6E).

Table 5. Expression of selected genes in leaves of silver birch, as affected by senescence. The mRNA levels of silver birch genes were quantified by reverse northern analysis. Signals on membranes were compared with the mRNA level of a constitutive gene, the translation initiator factor. The senescing leaf of long shoot from clone V5952 on DOY 275 was compared with the actively photosynthesizing leaf of short shoot on DOY 178. The relative abundance of transcript is denoted by -/+ when two to five-fold decrease/increase, --/++ when five to ten-fold, and ---/+++ when more than ten-fold decrease/increase was found. When weak signal was detected from both leaves studied, this is denoted by **ws**, but if signal was weak only in the other leaf sample the increase/decrease is shown and denoted by ws. No = no difference.

	DOY 178 leaf 0/ DOY 275 leaf 3
Photosynthesis	
RbcS, ribulose-1,5-bisphosphatase carboxylase/oxygenase, Y07779	--
Cab, chlorophyll a/b binding protein, est	---
Ca, carbonic anhydrase, est	---
Energy production	
G6PD, glucose-6-phosphate dehydrogenase, AJ279688	++
Mpt, mitochondrial phosphate translocator, Y08499	-, ws
Adh, alcohol dehydrogenase, AJ279698	+++, ws
Cell maintenance and development	
Lox1, lipoxygenase 1, AY124318	No
Lox2, lipoxygenase 2, AY124319	-
Lap, leucine aminopeptidase, Y14777	+
Cyp1, cysteine proteinase 1, AJ415385	++
Cyp2, cysteine proteinase 2, AJ415386	+
Atub, alpha-tubulin, AJ279695	---
Dad, defender against apoptotic cell death, AJ279687	-
Dhn, dehydrin	+
Antioksidants	
Apx, ascorbate peroxidase, AJ279686	-
Gpx, glutathione peroxidase, AJ279689	No
Gr, glutathione reductase, AJ279690	+
Gst, glutathione-S-transferase, tau	No
Gst, glutathione-S-transferase, theta, AJ279691	No
Cu/Zn sod, Cu, Zn superoxide dismutase, AJ279694	No
Cat, catalase, AJ295295	-
Defence genes	
Pr1, pathogenesis-related protein 1, AJ279696	No

	DOY 178 leaf 0/ DOY 275 leaf 3
Pr3a, acidic endochitinase, AJ279692	No
Ypr10, pathogenesis related protein 10, X77601	No
Wrky, DNA binding protein wrky, AJ279697	Ws
Hormone biosynthesis and perception	
Bp-ETR, ethylene receptor	No, ws
Bp-ACO1, ACC oxidase, Y10749	+
Bp-ACS1, ACC synthase, AY120897	Ws
Aos, allene oxide synthase, est	++
Phenylpropanoid biosynthesis	
DS3,3-deoxy-D-arabinoheptulosonate-7-phosphate	--
Pal, phenylalanine ammonium lyase, X76077	+
Chs, chalcone synthase, Y11022	---, ws

4.1.3 Late-senescing line R3.1 and wild-type clone R (III)

Photosynthesis and senescence were studied during development of lifespan leaves and in the youngest fully developed (yfd) leaves in a wild-type clone, R, and a transgenic line, R3.1. The amount and activity of Rubisco were stable in lifespan leaves of line R3.1, decreasing slightly on the last day of sampling (Figs. 4A and 4B in III). In contrast, in the lifespan leaves of clone R, the amount and activity increased along with leaf expansion (Figs. 2C, 4A, and 4B in III), and decreased thereafter. Also, the mRNA levels of *RbcS* decreased earlier in lifespan leaves of clone R than in line R3.1 (Fig. 5A in III). The maximal efficiency of PSII, Fv/Fm, measured from dark-adapted leaves, decreased in the lifespan leaves of clone R (Fig. 3A in III) but not in line R3.1. The actual efficiency of PSII in light, Fv'/Fm', increased along with expansion of the lifespan leaf in clone R, and decreased thereafter (Fig. 3B in III), while in line R3.1 the Fv'/Fm' was steady during the whole experiment.

The yfd-leaves which developed at the beginning of the experiment were larger than yfd-leaves that developed later (Fig. 2C in III). The decrease in leaf area was visible at the same time as height growth of plants ceased (Fig. 2A in III). Also, the amount and activity of Rubisco was lower in the youngest fully developed leaves of clone R later during the experiment than on the first two sampling days (Fig. 4A in III), and this was accompanied by decreased mRNA levels of *RbcS* in yfd-leaves of clone R. The maximal efficiency of PSII stayed high while the actual efficiency of PSII decreased in the yfd-leaves of clone R (Figs. 3A and 3B in III). In line R3.1, the yfd-leaves did not differ markedly from the lifespan leaves of R3.1 (Figs. 2C and 4 in III).

Gene expression was studied during active photosynthesis (day 23) and at early and advanced stages of leaf senescence (days 44 and 56) by reverse northern hybridization analysis in clone R (Table 3 in III). The results showed that mRNA levels of photosynthesis-related genes *RbcS*, *Cab*, and *Ca*, were down-regulated in the lifespan leaves on day 44 and continued to decrease on day 56 (Table 3 in III). In contrast, transcripts of several other genes became more abundant in senescing birch leaves: *G6PD*, *Mpt*, and *Adh* (energy production), *Lox1*, *Lox2*, *Cyp1*, *Cyp2* and *Lap* (cell maintenance and development), *Apx*, *Gpx*, *Gr*, *Gst*-tau, *Gst*-theta, and *Cu/Zn SOD* (antioxidants), *PR1*, *PR3a* and *Wrky* (defence genes), *Bp-ETR* (hormone biosynthesis and perception), and *DS3*, *Pal*, and *Chs* (phenylpropanoid

biosynthesis). The transcript level was so low in young leaf samples in the case of *Mpt*, *Adh*, *Lox1*, *Wrky*, and *Bp-ETR* that the magnitude of increase should be considered with caution (Table 3 in III).

Six genes were selected for RNA dot-blot hybridization analysis, which included leaf samples presenting various developmental stages from line R3.1 and clone R (Fig. 6 in III). Changes in mRNA levels of *RbcS* have been described above together with the other photosynthesis parameters measured. Messenger RNA levels of *Cab* followed the mRNA levels of *RbcS* in clone R, but in line R3.1 mRNA levels of *Cab* were as low as in senescing leaves of clone R (Figs. 6A and 6B in III). The amount of transcript of *Cyp1* increased markedly during leaf senescence in clone R and stayed at a low level in line R3.1 (Fig. 6D in III). The transcript of *Apx* was most abundant before the transcript levels of *Cyp1* increased in clone R (Figs. 6C and 6D in III). In line R3.1, the mRNA level of *Apx* stayed relatively constant in the leaves studied. Transcript levels of *Lox2* increased at the same time as those of *Cyp1* in the lifespan leaves of clone R, but an increase was also detected in the yfd-leaves on the last sampling day (Figs. 6D and 6E in III). Furthermore, in line R3.1 the transcript levels of *Lox2* increased even more on the last sampling day than those in clone R (Fig. 6E in III). *Pr1* mRNA levels increased only in advanced stages of leaf senescence in clone R (Fig. 6F in III).

4.1.4 Similarities and differences between greenhouse and field experiments

The time of reaching the highest photosynthetic capacity correlates with leaf expansion in silver birch (Figs. 1B and 2C in I, Figs. 2C, 4A, and 4B in III, Figs. 2, 4, and 5A). However, the declining rate was quite different in birch seedlings growing for their first year in the greenhouse, as compared to micropropagated silver birch plants during their seventh year under field conditions. In the greenhouse-grown seedlings (I) and R clone plants (III), the leaves developed at the beginning of the experiment were shaded by the new leaves that developed above them. Then photosynthetic capacity decreased in the lower leaves and the highest photosynthetic activity shifted into new leaves within two to three weeks (Figs. 1B, 2C, and 3 in I, Figs. 1B, 4A, 4B, and 6A in III). In young field-grown silver birches, photosynthetic activity decreased slowly in short shoot leaves and within 8 to 10 weeks the new long shoot leaves had higher photosynthetic activity than the short shoot leaves (Figs. 4, 5A, and 6A). The highest total activity of Rubisco recorded in the greenhouse, 16 μmol CO_2 $min^{-1}g^{-1}$ fw (Fig. 4B in III) was greater than in the field, 12 μmol CO_2 $min^{-1}g^{-1}$ fw (Fig. 4B). Also, the highest amount of Rubisco was clearly greater in the greenhouse, 25 mg g^{-1} fw, than in the field, 12 mg g^{-1} fw.

Generally speaking, the declining mRNA levels of SDGs correlated well with biochemical parameters, the activity and amount of Rubisco, both in greenhouse (Figs. 4A, 4B, and 6A in III) and in field experiments (Figs. 4B, 5A, and 6A). The amount of Rubisco protein and *RbcS* mRNA declined simultaneously also in seedlings grown in the greenhouse (Figs. 2C and 3 in I). Several leaf senescence-associated genes were identified but some differences were also observed among the greenhouse-grown seedlings (Fig. 4 in I), clone R (Fig. 6 and Table 3 in III), and the field-grown clones V5952 and K1659 (Fig. 6 and Table 5). Energy production-related genes *Adh* and *G6PD* appeared to be associated with leaf senescence in clone R and V5952, while *Mpt* was related to leaf senescence in the seedling and clone R (Fig. 4C in I, Table 3 in III). From cell maintenance and development-related genes, proteases (*Lap* and *Cyp*) and *Dhn* were related to leaf senescence in clone R and V5952 (Table 3 in III, Table 5). *Lap* was associated with leaf senescence in seedlings (Fig. 4D in I) and similarly for *Cyp1* in clone K1659 (Fig. 6B). *Loxs* and *Dad* were expressed during leaf senescence in cloneR but

not in V5952 (Table 3 in III, Table 5). Most of the antioxidant genes (*Apx*, *Gpx*, *Gst*, *Cu/Zn sod*) were expressed in senescing leaves of clone R (Table 3 in III) but not in clone V5952 (Table 5). Two antioxidant genes showed a similar trend between experiments: *Cat* was not related to senescence in either clone, while *Gr* was related to senescence in both cases. Furthermore, RNA dot-blot hybridization analysis showed that *Apx* mRNA levels were the highest already before the onset leaf senescence (Fig. 6C, Fig. 6C in III). Also, defence genes (*Pr1*, *Pr3a*, *Ypr10*, and *Wrky*) were expressed during leaf senescence in clone R (Table 3 and Fig. 6F in III) but not in clone V5952 (Table 5). *Ypr10* expression was related to leaf senescence in seedlings (Fig. 4B in III) and similarly for *Pr1* in clone K1659 (Fig. 6E). Hormone biosynthesis and perception genes were expressed differentially in clones R and V5952, and seedlings. *Bp-ETR* was related to senescence in clone R (Table 3 in III), and *Bp-ACO1* was related to senescence in both seedlings (Fig. 4A in I) and clone V5952 (Table 5). In phenylpropanoid biosynthesis, *Pal* was expressed both in clone R (Table 3 in III) and V5952 during leaf sensecence (Table 5) whereas *Chs* and *DS3* were expressed in clone R (Table 3 in III).

4.2 Production and cryopreservation of transgenic silver birch lines (II, IV)

Transformation efficiency based on transient GUS expression (Fig. 1 in II) varied among 29 *in vitro* callus lines (i.e. genotypes) originating from adult elite birch genotypes. There was no transformation vector-dependent effect on transient GUS expression when pRT99gus, pBI221.1, and pBI426 were compared between the six genotypes that were the most competent for biolistic transformation (II). These callus cultures were, however, not able to regenerate after transformation. For subsequent experiments, *in vitro* shoot cultures originating from dormant vegetative buds of adult trees and from twigs of one- and two-year-old plants were used. Precultivation of four to eight days before bombardment had a positive effect on transient GUS expression in *in vitro* stem pieces (II). For stable birch transformation, sense- and antisense-*RbcS* pRT99 vectors were constructed and, together with pRT99gus vector, were used in genetic transformation of *in vitro* silver birch clones. Only the lines derived from transformation with sense-*RbcS* pRT99 and pRT99gus, representing clones R and E5396, were able to regenerate. For some reason, no antisense-*RbcS* pRT99 lines were regenerated. The regeneration time was long and shoot formation from callus tissues started 10-13 months after transformation (II). Transformation frequency in the successful case with clone R was 6% (II), and with clone E5396 the frequency was about the same but exact percentage could not be determined due to difficulties in separating individual lines. For successful regeneration, cytokinin thidiazuron (TDZ) was needed to induce shoot formation, but BA was necessary for elongation growth of shoots (II).

Preservation of transgenic silver birch lines in liquid nitrogen, cryopreservation, was applied to vegetative buds of one- and two-year-old transgenic birch lines (IV). Two weeks after thawing, the regeneration of cryopreserved branch buds was lower than in the non-cryopreserved controls or cryopreserved stem buds, but after four weeks no significant differences were found (Fig. 3A in IV). The stability of transferred npt*II*-genes tested by Southern hybridization analysis was not affected by cryopreservation (Figs. 4A and 4C in IV). The mRNA levels of npt*II* showed no clear differences either before or after storage in liquid nitrogen (Figs. 4B and 4D in IV).

5 DISCUSSION

5.1 Leaf senescence in silver birch

Characterization of physiological events during leaf senescence in silver birch during a growing season has been conducted in field conditions with 40-year-old trees (Oleksyn et al. 2000). The results reveal that net photosynthesis is highest soon after full expansion of short shoot leaves and remains relatively high until a decline at the end of the growing season (Oleksyn et al. 2000). Ultrastructural changes in senescing birch leaves have shown that the volume of chloroplasts decreases and the size of lipid globules in chloroplasts increases (Dodge 1970). Ozone exposure also accelerates senescence, causing a decrease in net photosynthesis and Rubisco content and some structural changes including an increase in the size of plastoglobuli (Pääkkönen et al. 1996).

5.1.1 How can one define the stages of development?

Different stages of senescence have been determined based on chlorophyll content, as in *Arabidopsis* (Lohman et al. 1994) and *Brassica napus* (Buchanan-Wollaston 1997). However, the amount of Rubisco has proved to be the best indicator of photosynthetic capacity during senescence in soybean (Jiang et al. 1993). The actively photosynthesizing and senescing leaves used in identifying SAGs in silver birch were selected, based on the amount of Rubisco and the mRNA level of *RbcS* (III, Table 5). In clone R (III), it was possible to select leaves that represented the early and advanced stages of senescence, but in clone V5952 only an advanced stage of senescence was detected. Furthermore, in order to compare senescence-related changes, an actively photosynthesizing leaf was selected – but it was not known whether housekeeping metabolism such as lipid and protein turnover and the radical scavenging system were fully functional in these young leaves (III, Table 5.). This could partly explain the differences that were found between analysis of SAGs (III, Table 5).

5.1.2 Downregulation of photosynthesis

When net photosynthesis in mature leaves of silver birch has been followed until senescence, it has decreased from 15 to 5 μmol $m^{-2}s^{-1}$ in 2-year-old trees (Pääkkönen et al. 1996) and from 10 to 7 μmol $m^{-2}s^{-1}$ in 40-year-old trees (Oleksyn et al. 2000). During the present studies, net photosynthesis was measured in fully developed leaves in 2-month-old plants (Table I in III) and in still expanding long shoot leaves (Table 4) in 6-year-old silver birches. The measured values, 9,2 –9,9 μmol $m^{-2}s^{-1}$ (III) and 15,0-21,7 μmol $m^{-2}s^{-1}$ (Table 4) show that these leaves were actively photosynthesizing. The high net photosynthesis rate correlates well with the high amount of Rubisco (III, Fig. 5A). The senescence-related decrease in photosynthesis, based on amount and/or activity of Rubisco, started soon after the leaves were fully expanded, both in silver birch seedlings (I) and in clone R plants during their first growing season (III). A similar trend of decline in photosynthesis has been observed in *Arabidopsis* (Hensel et al. 1993) and soybean (Jiang et al. 1993). In the 6-year-old birches, the decrease in photosynthetic activity, measured as the amount and activity of Rubisco (Figs. 4, 5A), started in fully developed leaves and proceeded much more slowly than in seedlings (I) and clone R plants (III). The difference in photosynthesis decline might be related to the different lifespan of leaves during the first growing season and the following growing seasons. In silver birch plants growing for their first growing season, the increase in height and leaf development are

rapid under good greenhouse conditions. Then the lowest leaves become shaded by developed leaves above them, and the lowest leaves start to degrade Rubisco within a few weeks (I, III). The leaves of the short shoot burst from buds in spring and these leaves have lost some Rubisco protein 10 weeks after full expansion (Figs. 2 and 5A). Thus, it can be concluded that photosynthesis decreases in leaves of silver birch after the leaves are fully developed, but the rate of decline may vary due to growth habit or growth environment.

The maximal efficiency of PSII, Fv/Fm, has been shown to decrease in senescing leaves of barley (Humbeck et al. 1996), wheat (Lu and Zhang 1998) as well as in clone R silver birch (III) simultaneously with chlorophyll content. The actual efficiency of PSII, Fv'/Fm', decreased more than the maximal efficiency during leaf senescence both in barley (Lu and Zhang 1998) and in clone R silver birch (III). Light harvesting capacity was found to decrease at the same time with Rubisco LSU, but SSU decreased later in leaves of barley (Humbeck et al. 1996). In clone R, the capacity of PSII and the amount of Rubisco started to decrease simultaneously in the leaves studied (III); however, there might have been differences in timing of decline, but these were not noticed due to long intervals, 10 to 14 days, between the samplings and assays.

Loss of Rubisco protein as well as *RbcS* and *rbcL* trancripts occurs when the rate of photosynthesis starts to decrease in soybean leaves (Jiang et al. 1993). Furthermore, transcriptional control seems to regulate the amount of Rubisco. This tight correlation between Rubisco SSU and LSU and their transcripts has not been found in senescing leaves of barley (Humbeck et al. 1996) or bean (Bate et al. 1991) where the nuclear (SSU) and chloroplast (LSU) subunits decrease at different stages. In silver birch seedlings, *RbcS* transcript levels correlated well with the amount of Rubisco throughout leaf development (I). In clone R, the amount of Rubisco followed a similar trend to the mRNA level of *RbcS*, except on day 34 when the transcript level of *RbcS* was low but the amount of Rubisco was high (III). The amount of Rubisco is regulated by accumulation of *RbcS* transcripts (Cai-Zhong and Rodermel 1995). Possibly the high amount of Rubisco caused a lower transciption rate or mRNA stability of *RbcS* in the silver birch leaves studied. In silver birch clones K1659 and V5952, the trends in the amount of Rubisco and *RbcS* mRNA correlated quite well with each other (Figs. 5A and 6A).

Downregulation of photosynthesis was starting in line R3.1 lifespan leaves, which is based on the maximal efficiency of PSII, Fv/Fm, and the actual efficiency of PSII, Fv'/Fm', on the last two sampling days (III). Also, the amount and activity of Rubisco as well as the *RbcS* mRNA level decreased in lifespan leaves at the end of the experiment (III). These changes, together with the observation that the leaves of line R3.1 are yellowing, indicate that line R3.1 is able to senesce – although senescence was just starting during this experiment (III).

5.1.3 Senescence-associated genes (SAG) in silver birch

To date, a large number of senescence-associated genes have been identified from different herbaceous plants (Buchanan-Wollaston 1997). They encode proteases, lipases, and ribonucleases needed for catabolism, glutamine synthetase for nitrogen recycling, and PR-proteins, among many other genes (Buchanan-Wollaston 1997, Weaver et al. 1998). The genes that were investigated during leaf senescence in silver birch have been divided into subgroups relating to photosynthesis, energy production, cell maintenance and development, antioxidants, defence genes, hormone biosynthesis and perception, and phenylpropanoid

biosynthesis (III, Table 5) and the SAGs identified from the subgroups will be discussed below. These cDNAs and PCR-fragments of silver birch genes are not necessarily gene-specific, and it is possible that the gene expression presented reflects the mRNA levels of several gene family members. These senescence-related changes in gene expression and their possible role during leaf senescence are presented in Figure 7.

5.1.3.1 Energy production

Changes in gene expression related to energy production during leaf senescence have rarely been studied, especially in the case of *G6PD*, *Mpt*, and *Adh*. The mRNA levels of *G6PD* increased in birch (III, Table 5), and this could imply the activation of the pentose-phosphate pathway, leading to an increase in NADPH production. Also, *Mpt* transcripts became more abundant, indicating that more phosphate might be transported to mitochondria – probably to be used for ATP synthesis (I, III). The role of the increased trancript level of *Adh* is unknown (III, Table 5). Altogether, these changes in transcript levels of energy metabolism genes show that energy metabolism is changing during senescence. Further studies are needed in order to understand how these particular changes are related to the whole process of senescence.

5.1.3.2 Cell maintenance and development

Nitrogen mobilization from senescing leaves involves many different proteases (Huffaker 1990), (Feller and Fischer 1994). Increased mRNA levels of cysteine proteases have often been detected in senescing leaves (III, Fig. 6B, Hensel et al. 1993, Drake et al. 1996, Xu and Chye 1999, Ueda et al. 2000), whereas an increased mRNA level for *Lap* has been reported by our group for the first time (I, III, Table 5) The increase in *Lap*, *Cyp1*, and *Cyp2* mRNA levels in senescing leaves (I, III, Table 5) during loss of soluble proteins suggests that they participate in protein degradation. Some cysteine proteases are expressed only during senescence, such as SAG12 in *Arabidopsis* (Lohman et al. 1994). SAG12 is expressed from early stages of senescence (25% loss of chlorophyll) in *Arabidopsis* (Lohman et al. 1994); also, homologues of SAG12 in *Brassica* have a similar timing of expression (Noh and Amasino 1999). In some species, cysteine protease transcripts have also been detected in young leaves but they became more abundant during senescence when developmental stages were defined by leaf yellowing in *Solanum melongena* (brinjal) (Xu and Chye 1999), and in tomato (Drake et al. 1996). In silver birch, transcripts of *Cyp1* increased when the amount of Rubisco and the chlorophyll content started to decrease (III, Figs. 5A, 5B, and 6B). In general, it seems that the increased mRNA levels of cysteine proteases can be used as molecular markers of leaf senescence.

Lipoxygenase has a role in lipid degradation (Thompson et al. 1998) and it also produces substrate for jasmonate biosynthesis (Schaller 2001). The increase in mRNA levels of *Lox1* and *Lox2* in clone R (III) and in mRNA level of *Lox2* in clone V5952 (Fig. 6D) could indicate increased membrane turnover in mature leaves and production of substrate for jasmonate biosynthesis.

The expression of dehydrin (*Dhn*) and defender-against-apoptotic cell death (*Dad*) should be studied in more detail during leaf development before they can be classified as senescence-associated genes in silver birch. Accumulation of dehydrin mRNA is related to cold and drought stresses in spruce (Richard et al. 2000). The small increase in the mRNA level of *Dhn* in leaves of silver birch clones R and V5952 (III, Table 5) may be related to the dry growing season of 1997 (Fig. 2) in the case of clone V5952. However, the increase in the level of *Dhn* transcript in clone R cannot be explained by drought. The mRNA level of *Dad* was

downregulated during petal senescence (Orzáez and Granell 1997) and during leaf senescence in clone V5952 (Table 5). In contrast, the mRNA level of *Dad* increased slightly in clone R leaves (III).

5.1.3.3 Antioxidants

The balance between ROS production and scavenging changes during leaf senescence. The activities of individual antioxidative enzymes have shown distinct patterns of enzyme activity during natural leaf senescence (Hodges and Forney 2000, Kanawaza et al. 2000)). As an example, the total activity of SOD has been seen to decrease in leaves (Hodges and Forney 2000, Klessig et al. 2000, Shrivalli and Khanna-Chopra 2001), but activity of SOD increased in isolated chloroplasts and peroxisomes (Pastori and del Río 1994, Pastori and del Río 1997). Additionally, the activity of FeSOD was found to increase in mitochondria (Shrivalli and Khanna-Chopra 2001) during natural senescence. In some studies, the transcript levels of antioxidant genes have been investigated during senescence; *Apx* increased in tomato (Gadea et al. 1999), *Gst* in carnation (Itzhaki et al. 1994), *Gst* (tau-class) in potato (Droog 1997), while *cat1* increased and *cat2* and *cat3* decreased in pumpkin (Esaka et al. 1997), and *sodB* and *sodCp* decreased in tobacco (Kurepa et al. 1997). In silver birch, the mRNA levels of antioxidant enzymes *Apx*, *Gpx*, *Gr*, *Gst*-tau, *Gst*-theta, and *Cu/Zn Sod* increased in clone R leaves (III) while *Cat* mRNA levels stayed unchanged. On the contrary, in the field-grown clone V5952 only the mRNA of *Gr* became more abundant and the mRNA levels of *Apx* and *Cat* decreased (Table 5). More detailed studies (III, Fig. 6C) revealed a similar trend in accumulation of *Apx* transcripts during leaf development in clones R, K1659, and V5952. Part of the conflicting results observed in different species is due to the fact that sampling has been carried out at different stages of leaf senescence. Furthermore, more information about function of antioxidative enzymes and ROS production in different cell compartments might reveal more similarities. There might also be differences between species in temporal function of antioxidant enzymes and ROS production during senescence.

5.1.3.4 Defence genes

Many defence-related genes are induced during leaf senescence (Quirino et al. 1999, Quirino et al. 2000). For instance, the transcript levels of *PR10* (Crowell et al. 1992), and *PR-1a-like* (Hanfrey et al. 1996) – which are individual members of the PR-proteins – increased during senescence, and chitinases show increased protein content (Lers et al. 1998). In silver birch, the transcript levels of *PR1*, *PR3a* and *Ypr10* increased in clone R plants (III) and the transcript level of *PR1* increased in clones K1659 and V5952 (Fig. 5E). The role of PR-proteins during leaf senescence as protection against pathogens has often been suggested. The necrotic lesions have developed faster in old than in young leaves after inoculation with incompatible and compatible strains of bacteria, showing enhanced protection in senescing leaves of tobacco when an increased mRNA level of *PR1* was also observed (Obregón et al. 2001). However, the old leaves were not capable of restricting the spread of compatible pathogen and this does not support the idea of improved defensive potential during senescence (Obregón et al. 2001). Generally, the function of PR-proteins during senescence will remain unknown until the specific functions of these proteins are recognized and the physiological significance during senescence is confirmed. The expression of PR-proteins can be regulated via salicylic acid-dependent pathways (Pieterse and van Loon 1999), ROS (Surplus et al. 1998), ethylene (Coupe et al. 1997), and sugars (Hebers et al. 1996), which all are involved in regulation of leaf senescence.

The WRKY transcription factors regulate pathogen and stress responses, but some members are also involved in regulation of leaf senescence (Eulgem et al. 2000, Hinderhofer and Zentgraf 2001, Robatzek and Somssich 2001). The *Wrky* of silver birch is most identical to At*WRKY48*, the function of which has not been reported in detail. The increased mRNA level of transcription factor *Wrky* in clone R leaves (III) suggests that it may participate in regulation of gene expression during leaf senescence.

5.1.3.5 Hormone biosynthesis and perception

Transcripts of ACC-oxidase have been shown to be abundant in senescing leaves of tomato (John et al. 1995) but more detailed studies showed that only some members of the ACC-oxidase gene family were accumulating in senescing tomato (Barry et al. 1996) and *Nicotiana glutinosa* (Kim et al. 1998) leaves. Transcriptional activation of ACC-oxidase has been detected during leaf senescence, with ethylene being formed (Blume and Grierson 1997). In the present study, accumulation of *Bp-ACO1* mRNA was highest at the onset of leaf senescence in silver birch seedlings (I), decreasing in the oldest leaves as was also found in tomato (Barry et al. 1996). There was also a small increase in mRNA levels of *Bp-ACO1* in clone V5952 (Table 5), but no change in clone R (III). Although ethylene is probably produced in senescing leaves of silver birch, it is questionable whether transcript levels of *Bp-ACO1* correlate with ethylene production. Accumulation of mRNA for putative ethylene receptors has been studied in tomato (Lashbrook et al. 1998), where two members of this gene family, *Le-ETR2* and *NEVER RIPE* (*NR*), were found to be downregulated in senescing leaves. Further studies showed that ethylene receptors were negative regulators, and they were able to compensate for the reduced mRNA level of *NR* but not *Le-ETR4* by expression of another family member (Tieman et al. 2000). The *Bp-ETR* mRNA in birch was visible at the early stages of leaf senescence but decreased during advancing senescence (III, Table 3). This might reflect increased sensitivity to ethylene at a later stage of senescence but the transcript levels of *Bp-ETR* were so low that the differences found should be verified with a more sensitive method.

Allene oxidase synthase (AOS) regulates octadecanoid biosynthesis in *Arabidopsis* (Laudert and Weiler 1998). When the promoter of *Aos* was fused with GUS, the activity of GUS was found in senescing leaves of *Arabidopsis* and tobacco (Kubigsteltig et al. 1999). The increased transcript level of *Aos* in senescing leaves of clone V5952 (Table 5) might suggest activation of octadecanoid biosynthesis and production of jasmonates. Since transcript levels of *Lox2* increased in mature leaves (Fig. 6D), substrates for octadecanoid biosynthesis were available when the leaves started to senescence. These senescing leaves of silver birch might also produce ethylene and salicylic acid which would induce the mRNA levels of *Aos* as well as the activity of AOS (Laudert and Weiler 1998). The function of jasmonates during senescence is unclear, but they are known to activate the expression of *Pal* and *Chs* and to depress expression of photosynthetic genes (Creelman and Mullet 1997).

5.1.3.6 Phenylpropanoid biosynthesis

Transcript levels of *Pal* and *Chs* increased in leaves of clone R during senescence (III), but only the transcripts of *Chs* became more abundant in leaves of clone V5952 (Table 5). This indicates activation of the phenylpropanoid and flavonoid pathways since PAL is the rate-limiting enzyme of the phenylpropanoid pathway and CHS is the first enzyme of the flavonoid pathway (Hahlbrock and Scheel 1989). Some products of the phenylpropanoid and flavonoid pathways have been found to be involved in leaf senescence; products of phenolic acid metabolism functioned as natural antioxidants (Tamagnone et al. 1998) and salicylic acid

regulated gene expression (Morris et al. 2000). In addition, in aging leaves of a tropical tree (*Schefflera arboricola*) two major flavonoids, quercetin and kaempferol glycosides, scavenge $H_2O_{2,}$, but their levels were found to decrease during senescence (Yamasaki et al. 1997). In senescing tree leaves, accumulation of anthocyanins into vacuoles has been proposed to have a photoprotective role during loss of chlorophyll when remobilization of nutrients occurs (Hoch et al. 2001).

5.1.3.7 Concluding remarks

Gene expression studies during silver birch leaf senescence have shown that when photosynthetic activity decreases, the mRNA levels of several genes increase (Figure 7). Transcripts of proteases (*Lap*, *Cyp*) became more abundant, which suggests that they may be involved in protein mobilization. Breakdown of lipids during senescence produces substrate for lipoxygenase (*Lox*), which in turn produces substrate for the octadecanoid pathway. The mRNA level of the key enzyme of the octadecanoid pathway, *Aos*, increased and this can lead to production of methyl jasmonate, which can regulate gene expression. Besides methyl jasmonate, ethylene and salicylic acid can also regulate gene expression during senescence. Based on increased transcript levels of *Bp-ACO1* and *Pal*, production of these hormones may be increased. The increased phenylpropanoid biosynthesis may also lead to production of flavonoids, which can participate in photoprotection in senescing leaves.

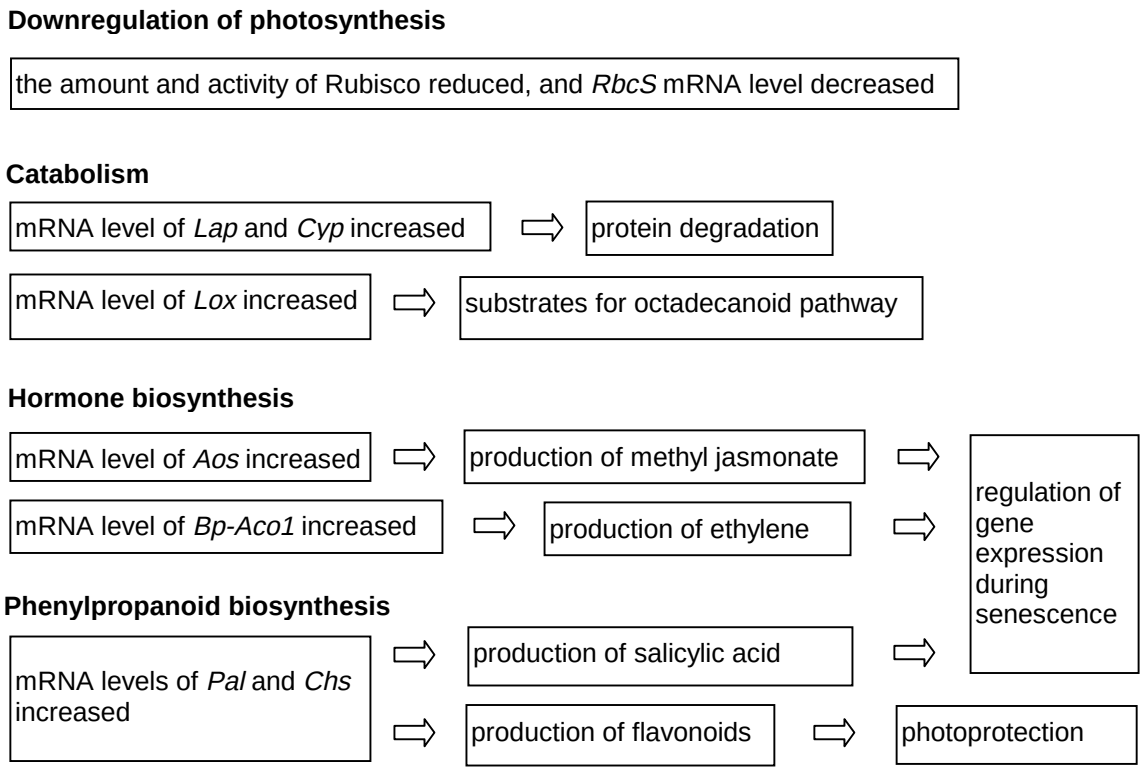

Figure 7. Senescence-related changes in the leaves of silver birch and their potential role during senescence

5.2 Production and cryopreservation of transgenic silver birch lines

Several aspen and poplar species from the genus *Populus* have been among the first transgenic trees produced (Klopfenstein et al. 1997). During the past decade, both *Agrobacterium*-mediated and direct biolistic transformation have been applied successfully in several other broad-leaf tree species also, such as English elm (*Ulmus procera*) (Gartland et al. 2000), sour orange (*Citrus aurantium*) (Ghorbel et al. 2000), eucalypt (*Eucalyptus camalduensis*) (Harcout et al. 2000), apple (*Malus x domestica* Borkh.) (De Bondt et al. 1994), white birch (*Betula platyphylla* var. *japonica*), and silver birch (Keinonen-Mettälä et al. 1998, II). Although the transformation frequency achieved by current transformation methods is still quite low with chestnut (1,5-1,8%) (Seabra and Pais 1998) and apple (0,4-4,6%) (Yao et al. 1995), higher frequencies have been achieved with English elm (7%) (Gartland et al. 2000) and sour orange (7%) (Ghorbel et al. 2000), and hybrid aspen (Klopfenstein et al. 1997) (around 20%). In silver birch, the transformation frequency was 6% with biolistic transformation (II), while for white birch it was reported to be 18% with *Agrobacterium*-mediated transformation (Mohri et al. 1997). Generally, both biolistic and *Agrobacterium*-mediated transformation techniques require optimization for each species genotype and tissue type. In the biolistic method, the physical parameters such as size of the particles, pressure and distance from the target tissue must also be tested. With the *Agrobacterium*-mediated method, host specificity, density of bacteria, and co-cultivation time must usually be optimized. The improvements in transformation frequencies have resulted for instance from selecting the most suitable genotypes as explants (De Bondt et al. 1994, II) and selecting the most appropriate explant type and developmental stage (De Bondt et al. 1994, Ghorbel et al. 2000, II). Precultivation before transformation has turned out to be effective for increasing transient GUS expression in birch (II) and apple (De Bondt et al. 1994). Furthermore, different hormone combinations in growth media used for regeneration have been shown to be worth testing. Synthetic cytokinin thidiazuron has been used in tissue culture media for woody plants (Huetteman and Preece 1993). Thidiazuron enhanced shoot formation but inhibited their elongation in transformed silver birch (II), as well as in *Populus tremula x P. tremuloides* (hydrid aspen) (Nilsson et al. 1992). However, only a positive effect of thidiazuron was found on shoot regeneration and growth in English elm (Gartland et al. 2000), *Populus alba x P. grandiata* cv. 'Crandon' (hybrid poplar) (Howe et al. 1994), and *Populus tremuloides* Michx (quaking aspen) (Tsai et al. 1994).

The regeneration time for transgenic shoots has varied from four months in white birch (Mohri et al. 1997), silver birch (Keinonen-Mettälä et al. 1998), and hybrid aspen (Klopfenstein et al. 1997) up to 18 months in English elm (Gartland et al. 2000). In the present study, the shoot regeneration step required from 9 to 12 months for clone R (II). The variation in regeneration time could be due to different cultivation conditions or different silver birch clones used for transformation. Keinonen-Mettälä et al. (1998) have used a higher thidiazuron concentration than was applied in this study (II). However, in some species such as white birch, thidiazuron was not necessary for successful regeneration (Mohri et al. 1997), which underlines the importance of optimizing the regeneration conditions individually for each species and clone, as observed during this work.

5.2.1 Late-senescing silver birch mutant

The dwarf line R3.1 was generated during transformation work with the sense*RbcS*-construct. The analysis of both *RbcS* mRNA level and amount of Rubisco protein (III) showed no reduction in mRNA or protein when compared to leaves of the wild-type line R. It was therefore concluded that line R3.1 is most probably a consequence of gene disruption by

inserted genes (III). There are multiple copies, at least six, of sense-*RbcS* contruct present, based on *nptII* and *RbcS* Southern hybridization analysis (II). Our preliminary analyses (data not shown) suggest that these might have been integrated only in one or two positions in the genome of line R3.1, and therefore disruption of one or two genes might have caused this phenotype. The mutant line R3.1 showed a delay in leaf age-dependent senescence when compared to lifespan leaves of wild-type clone R which had developed at the same time. Late-senescing varieties are found within maize, sorghum, fescue, and rice species (Thomas and Smart 1993) and further studies have given new information about senescence (Vicentini et al. 1995, Kingston-Smith et al. 1997).

5.2.2 Cryopreservation

The cryopreservation method of *in vivo* cold-hardened buds of silver birch (Ryynänen 1996, Ryynänen 1999) was applied successfully to transgenic silver birch lines that were grown in the greenhouse (IV). Transgene stability has been observed in roots of *Nicotiana rustica* and *Beta vulgaris* (Benson and Hamill 1991) and in cells of *Papaver somniferum* (opium poppy) (Elleuch et al. 1998), as well as in leaves of different transgenic lines from two silver birch elite genotypes, R and E (IV). The transferred *nptII* genes have been found to be functional after cryopreservation in opium poppy cells, where the mRNA level of S-adenosyl-L-methionine synthetase was maintained (Elleuch et al. 1998), and in wheat cells where NPT activity was maintained (Fretz and Lörz 1995). In the present study, the mRNA levels of *nptII* genes varied between control and cryopreserved birch lines, but cryostorage had no clear effect on the levels of transcript (IV). Plant regenerated from cryopreserved branch bud of line R7.2 had less *nptII* mRNA than the control plant, but this may have been due to individual variation between plants – and the collected leaves were not necessarily at the same stage of development. This report (IV) shows the possibility of preserving transgenic woody plants by means of cryopreservation.

6 CONCLUSIONS

Leaf senescence in silver birch seedlings, and clone R plants grown in the greenhouse for their first growing season (I, III), showed that senescence proceeds in an orderly manner resembling the sequential senescence found in tobacco. The lowest and the oldest leaves senesced first, while the newly developing leaves continued to photosynthesize. The 6-year-old birch trees from clones K1659 and V5952 growing in the field showed that high photosynthetic capacity was maintained for a long time during the growing season in short shoot leaves, before it started to decrease. Clear senescence-related loss of photosynthetic capacity was found late during the growing season in leaves of the long shoot. Generally, leaf senescence in silver birch proceeds in a well-organized manner: when the amount of Rubisco decreased, the transcripts of cysteine protease became more abundant in senescing leaves. Several senescence-associated genes that have been described earlier in herbaceous plants were identified in silver birch. Furthermore, new putative SAGs such as *Lap* and *Mpt* were found.

The biolistic transformation method was developed to produce transgenic silver birch lines for research on carbon metabolism. A late-senescing silver birch line R3.1 was found among the transgenic sense-*RbcS* lines. Line R3.1 is the first late-senescing mutant to be described from tree species so far, and it was further characterized in a greenhouse experiment. Leaves of line R3.1 maintained active photosynthesis longer than leaves of the wild-type clone R. The cryopreservation method used turned out to be successful for maintaining interesting and important transgenic silver birch lines, and both the stability and function of the transferred *nptII* genes were maintained.

7 FUTURE PROSPECTS

The progression of leaf senescence within different cell compartments requires further studies. Also, more information about environmental and internal signals and their interaction and regulation in leaf development is needed before we can understand the process of senescence properly. In the present study, senescence-associated genes were identified in silver birch from a group of genes isolated during different stress conditions. The est (expressed sequence tags)-technology has been applied in silver birch when cold-stress and ozone-stress related genes have been identified. Est-technology could also be used to identify senescence-related genes in silver birch. Alternatively, the existing est-sequences might be collected onto DNA-chips and a mRNA pool from senescing silver birch leaf could be used to find out which genes are expressed during senescence. Furthermore, how the genes that are expressed during senescence participate in cell metabolism must also be investigated.

The late-senescing line R3.1 can be used in further studies to identify gene(s) that affect growth and leaf senescence in silver birch. Also, the function(s) of disrupted gene(s) during leaf development must be characterized. Furthermore, the methods used for production and preservation of transgenic silver birch lines in the present studies can be used in future research work.

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