



Regulatory Control of Autumn Senescence in *Populus tremula*

Erik Edlund



Umeå Plant Science Center
Department of Plant Physiology
Umeå 2016

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To Frida and Emily

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Abstract

Autumn senescence is a visually spectacular phenomenon in which trees prepare for the oncoming winter. The mechanism for regulation of autumn senescence in trees has been very hard to pinpoint. In this thesis the main focus is to investigate how autumn senescence is regulated in aspens (*Populus tremula*).

Previous work has established that autumn senescence in aspens is under daylight control, in this thesis the metabolic status and the effect on autumn senescence was investigated. The metabolic status was altered by girdling which leads to accumulation of photosynthates in the canopy. This resulted in an earlier onset of senescence but also the speed of senescence was changed. At the onset of senescence the girdled trees also accumulated or retained anthocyanins.

The nitrogen status of aspens during autumn senescence was also investigated, we found that high doses of fertilization could significantly delay the onset of senescence. The effects of various nitrogen forms was investigated by delivering organic and inorganic nitrogen through a precision fertilization delivery system that could inject solutes directly into the xylem of the mature aspens. The study showed that addition of nitrate delayed senescence, addition of arginine did not have any effect on the autumn senescence in aspens, and furthermore the nitrate altered the trees leaf metabolism that was more profound in high dosages of supplied nitrate.

Cytokinins are plant hormones believed to delay or block senescence, studies have suggested that the decrease of cytokinins and/or cytokinin signalling may precede senescence in some plants. To investigate how cytokinin regulates autumn senescence in aspens we profiled 34 cytokinin types in a free growing mature aspen. The study begun before autumn senescence was initiated and ended with the shedding of the leaves, and spanned three consecutive years. The study showed that the individual cytokinin profiles varied significantly between the years, this despite that senescence was initiated at the same time each year. Senescence was furthermore not connected to the depletion of either active or total cytokinins levels. The gene pattern of genes known to be associated with cytokinin was also studied, but no gene expression pattern that the profile generated could explain the onset of senescence. These results suggest that the depletion of cytokinins is unlikely to explain the tightly regulated onset of autumn leaf senescence in aspen.

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Abbreviations

All abbreviations are explained when they first appear in the text.

List of Publications

This thesis is based on papers described here below which will be referred to by their Roman numerals in the text

- I. **Erik Edlund**, Lars Björken, Kathryn Robinson & Stefan Jansson (2016). Girdling Influence Onset of Senescence and Anthocyanin Expression in Aspen. (*Manuscript*)

- II. **Erik Edlund**, Lars Björken, Ulrika Ganeteg, Torgny Näsholm & Stefan Jansson (2016) Nitrate, but not arginine, influence aspen autumn phenology (*Manuscript*)

- III. **Erik Edlund**, Ondrej Novak, Karin Ljung & Stefan Jansson (2016). Contrasting patterns of cytokinins between years in senescing aspen leaves. (*Accepted with minor revision in Plant Cell & Environment*)

Introduction

Senescence

Senescence is for most of us the spectacular yellowing of leaves and the obvious colour changes the plant undergoes in the later stages of leaf senescence. Since senescence means to grow old in Latin the connection to the late developmental stages in plants is obvious. Senescence is a highly regulated process and conducted in an orderly fashion, it is not the same as necrosis but differs in that the cell organs are deconstructed and much of the content reallocated (Woo *et al.*, 2013). When the plant cell receive the initiation signal for starting the programmed cell death (PCD) program, the cell starts to dismantle itself in a very regulated manner starting with the chlorophyll molecules, the nucleus and mitochondria that are vital for the cells function are the last organelles to be dismantled (Thomson and Plat-Aloia 1987; van Doorn and Woltering 2004). That the most vital organelles are dismantled last is very indicative of that the cell needs to remain functional during senescence. The last event of the cell is the rupture of the vacuole and plasma membrane disruption which leads to the cells death. PCD and senescence are in essence similar processes but the PCD terminology is mostly used when it is discussed on a cellular level and senescence when the describing PCD on a macro scale such as in leaf senescence. The word apoptosis, which characterizes one type of PCD (dependent on specific proteases, caspases, and an active involvement of the mitochondria) that has been much studied in animal systems, is actually derived from the greek expression for falling autumn leaves. However, it appears as if this type of cell death is not prominent in plants, despite the name. Once the leaf has entered senescence the photosynthesis is gradually stopped and the chloroplast is the first cellular organ to begin dismantling followed by the remaining non-vital organs that are deconstructed and remade into more easily transportable forms (Nooden 1988). The ability for plants to use senescence as a mean to regain valuable nutrients from leaves is a feature that is highly beneficial for the plant, these resources would otherwise be lost to the if they were not orderly transported out from the leaf before being shed. The plants have invested several important nutrients in the leaves, primarily nitrogen but also many macronutrients (i.e. phosphor, sulphur, potassium) and micronutrients (i.e. Fe, Zn, Cu, Ni, Mo, B, Cl) (Himelblau and Amasino 2001; Hörtensteiner and Feller 2002). The chlorophyll is dismantled early in the senescence process mainly to safeguard the leaf from potential damage caused by uncoupled chlorophyll molecules that would create reactive oxygen species and thereby oxidative damage if the downstream reactions of photosynthesis are not operating (Hörtensteiner *et al.*, 2006; Hörtensteiner 2009). The nitrogen in chlorophyll has been thought to be a source for nitrogen recycling in senescing leaves (Hendry *et al.*, 1987). But recent data seems to indicate that the chlorophyll bound nitrogen is not actually recycled but the dismantled chlorophyll molecules are left in the leaf as nonfluorescent

chlorophyll catabolites (Hörtensteiner and Kräutler 2011). The nutrients are transported out of the leaves during senescence towards the sink part of the plant, in the case of trees they are transported to the bark, twigs and stem and some to the roots (Cook and Weih 2005).

Plants or leaves that enter senescence are related to reaching a certain developmental age, but also external and environmental factors can initiate senescence (Lim *et al.*, 2007). Many plants undergo senescence when they have reached the final developmental stage. Plants need to know when to enter senescence to not undergo this process too early. To solve this, plants have poorly understood age related factors also called age related changes (ARC) an ARC can be a developmental stage that is irreversible such as the end of leaf expansion or cell division (Jibrán *et al.*, 2014). Studies in *Arabidopsis* have shown that it can't enter senescence before a certain age of the leaves has been reached (Jing *et al.*, 2002; 2005). This ARC prerequisite stops young leaves from senescing and acts as a failsafe for not initiating the senescence program in the wrong developmental timeframe (Weaver *et al.*, 1998). One very obvious ARC in plants is the leaves capacity to conduct photosynthesis (Thomas and Howarth 2000). This is illustrated by Nooden *et al.*, 1996 who exposed leaves to an excessive amount of light which hastened the onset of senescence. This phenomenon of age induction can actually be reversed, some monocarpic plants such as chickpea senescing leaves will reverse into a juvenile state if the reproductive organs are removed, since the sink of the flower or seeds have disappeared (Flowers 2009).

Many factors in addition to age can initiate senescence in plants, for example abiotic stresses such as salinity, drought, wounding, shading, nitrogen deficiency and heat (Gan and Amasino 1997). Biotic factors is also known to induce leaf senescence as defence strategy or simply by the damage inflicted, all these different initiation pathways have a specific genetic profile connected to each specific induction factor (Chen *et al.*, 2002). Senescence can be divided into four initiation subgroups that at a developmental level exploit senescence in slightly different ways. It should be noted that the programmed senescence are in most cases not reversible and will be completed regardless of external cues, some senescence's (e.g. dark induced senescence) can be reversed if the condition that initiated the senescence are removed or relived before a certain point in its progression (Stoddart and Thomas 1982). The nature of the "point of no return" is unknown.

Whole plant senescence is for many annual seed producing plants a way to remobilize as much nutrients as possible from the vegetative parts and reinvest them in the seeds at the end of the lifecycle, this type of senescence can be found in plants like wheat and rice and other annuals that end their life cycle with seed production. It has been shown that the majority of the nutrients that are needed to create seeds will be taken from the senescing tissue (Feller and Keist 1986).

Sequential senescence is progressive form of senescence of the older leaves and lateral organs. This type of senescence is used to remobilize nutrients from the older leaves as the shoot continuously produced new ones, which has a better capacity to contribute to photosynthesis. Shoot senescence is used in many herbaceous biennials and perennials such as banana, onions and some root fruits, they only senescence the above ground parts and store the remobilized nutrients in the roots and bulbs below ground.

There are also some “local types” of leaf senescence that can be triggered in individual leaves. For instance if a plant leaf that is heavily shaded underneath a canopy or it might be one of the first leaves to appear on a small herbaceous plant, then the efficiency of the leaf will be severely decreased in the late stages of the growth cycle. Therefore it is advantageous to use the invested nutrients in the unproductive shaded leaf and remobilize them into new tissue in a more favourable location. A partially shaded leaf will enter a fast progressing senescence (Weaver and Amasino 2001; Keech *et al.*, 2007). Leaves that are completely darkened don't senescence as fast (Mae *et al.*, 1993). These types of senescence are referred to as dark induced senescence (Rousseaux *et al.*, 1996).

When leaves are attacked by insect herbivores or fungi it might trigger a stress-induced senescence that is localised to the attacked parts. The plant then actively sacrifices the leaf before the attack can completely destroy it and potentially spread and thereby regain some of the investment from the leaf, this type of senescence is often local to the affected leaf or parts of the infected leaf (Smart *et al.*, 1994).

Synchronous senescence like autumn senescence is an event that is conducted simultaneously in the whole canopy of the plant, usually in deciduous trees in temperate zones that will need to shed their leaves for the oncoming winter or risk losing the leaves to frost (Näsholm *et al.*, 1998).

Autumn Senescence in trees.

We who live in the temperate parts of the world can enjoy the yellowing and reddening of leaves every autumn (Lee *et al.*, 2003). The visually astonishing phenomenon known as autumn senescence also have a crucial role to fill in the long term survival of trees (Ghelardini *et al.*, 2014). The striking colours that autumn brings are caused by chlorophyll degradation that will bring out the bright colours of carotenoids and anthocyanins for us to see. Autumn senescence in a single tree takes often a couple of weeks depending on environmental factors (Fracheboud *et al.*, 2009). Autumn senescence in many trees depends on the seasonal variation in temperature to initiate (Richardsson *et al.*, 2009), although some trees uses the changes in day light as the initiating factor (Fracheboud *et al.*, 2009; Keskitalo *et al.*, 2005). Trees usually undergo senescence out of necessity, the option not to

remobilize the nutrients of the leaves would most likely weaken the tree and it could succumb to either abiotic or biotic factors more easily (Näsholm *et al.*, 1998). An interesting note to this is that there is several tree species that do not undergo senescence, even though they grow under similar conditions. For example alders (*Alnus sp.*) that can grow on soils with very low nitrogen content, as they have a nitrogen fixing symbiont (*Frankia*). This in turn gives the alders the option to not undergo autumn senescence and instead focus on carbohydrate assimilation and let the leaves fall off with the first frost (Taulavuori 2006). Conifers have adapted a hibernation strategy for their needles and do not shed their needles in late autumn but rather when they have reached a certain age (which could be many years), but instead prepare them for winter survival by infusing them with sugars and other frost tolerance substances (Ögren *et al.*, 1997). If these alternatives exist then why do trees shed the leaves at all? Even though the tree will lose valuable resources by shedding the leaves one might consider that the overall construction of many leaves is made in a way that they aren't really supposed to function at peak efficiency for more than one season, Over time, the leaf will accumulate damage and deleterious compounds that no longer function and will just be left inside the leaf and be discarded when it is shed (Buchanan-Wollaston 1996). The relative carbon cost of constructing new leaves in the spring is not that high (Dicksson 1998), compared to evergreens that keep their leaves and need to spend a higher percentage of the carbon gain towards maintenance (Percy *et al.*, 1987).

Onset of senescence.

The concept of onset of senescence is defined as the change in the metabolic state of the leaf from being fully photosynthetically active to a senescing state where the leaf is actively broken down into valuable components that are transported out of the leaf (Fisher and Feller 2002). This change is easiest to follow on a larger scale by measuring chlorophyll since the degradation of this molecule is one of the first visible markers in the senescence chain of events (Ougham *et al.*, 2008; Matile 1992). Although once the decrease in chlorophyll is obvious for the eye, the actual onset event has already occurred as shown by studies conducted by Hinderhofer 2001 and He *et al.*, 2001. When senescence has been initiated regardless of initiation pathway it seems that the execution of senescence follows the same set path (Guo and Gan 2012). Since the actual molecular triggers are still poorly understood we cannot with certainty say what exactly occurs in the moment of change, only the state of the leaf before and after (Buchanan-Wollaston 1997; Quirino *et al.*, 2000). Much previous work has been focused on removing as many variables in this event as possible to eliminate possible noise that might obscure the actual trigger event. Our understanding of the factors governing the circumstances that are needed for initiation of senescence has increased, although the actual trigger has been shown to be much more complex than first assumed.

Senescence in aspens

Autumn senescence have been studied intensively in aspens trees (Keskitalo *et al.*, 2005; Bhalerao *et al.*, 2003; Andersson *et al.*, 2004; Fracheboud *et al.*, 2009) and these studies have provided a good knowledge base for this work. Young aspens often grown and form leaves throughout the summer but big aspens in our climate typically only have one flush, i.e. all leaves are formed from winter buds that was developed in the previous autumn and these do not initiate senescence simply because the leaves have reached a certain age, but before it is capable of initiating autumn senescence the tree needs to go through growth arrest and set bud (Fracheboud *et al.*, 2009). It is very well established that bud set is controlled by daylength, see e.g. Howe *et al.*, 1996. The bud set appear to start at a similar date every year regardless of external environmental factors and are controlled by for example phytochromes (Olsen *et al.*, 1997). Before the buds have set the tree cannot enter autumn senescence by the normal cues, the trees needs to acquire a “competence to senescence”. Once the buds have set triggered by photoperiodic cues there seems to be a phase of 10-30 days (clone dependent) until the tree have achieved a competence to senesce. Once a tree has achieved the competence to senesce it is susceptible to the (so far unknown) environmental cues that can initiate it (Fracheboud *et al.*, 2009). The capacity to use daylight as initiation factor for autumn senescence might be of higher value in climates with fast changing seasons and with daylength changing up to an hour a week in the most northern latitudes it gives the trees a very accurate perception of the date and more reliable at a much higher resolution than a drop in temperature could provide. The cold hardiness that aspens develop for the oncoming winter seems not to be connected to initiation of senescence but rather with the bud set (Michelson *et al.*, submitted, *Figure 1*). Full freezing tolerance seems to be acquired after a prolonged low temperature cue as shown by Welling *et al.*, 2002. If the environmental cue is not given it seems that the tree will continue to stay photosynthetically active until the ARCs accumulate and initiate senescence, experiments conducted by Fracheboud *et al.*, 2009 were aspens were grown under a 21h light period that was prolonged and kept until September 21, the experiment showed that not only was the trees inhibited from initiating senescence but a clear correlation could be seen between late sensing trees and the speed of senescence that increased with trees starting senescence late. It also seems that aspen trees that grow in more northern latitudes have a stricter genetic control of the initiation and start the senescence earlier than trees that grows in the southern latitudes (Fracheboud *et al.*, 2009). Interestingly, the daylight signal that initiate onset on senescence seems not to be daylength per se, but some other, yet unidentified, daylight related factor (Michelson *et al.*, submitted).

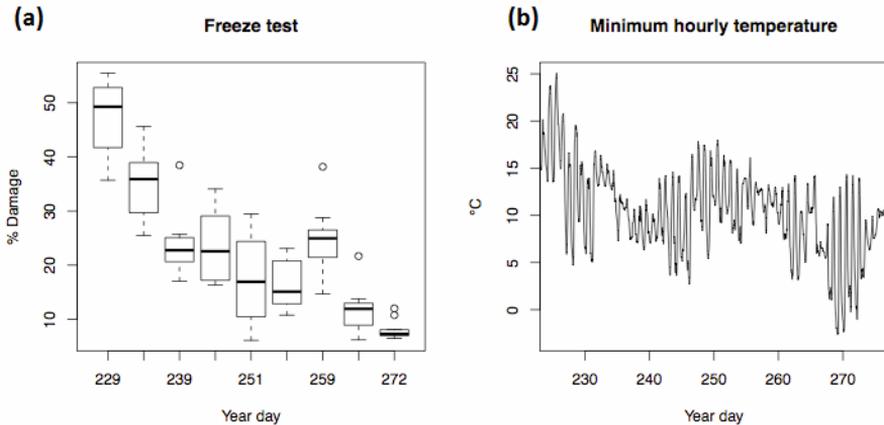


Figure 1: The initial phase of cold hardiness development is coordinated with bud set. a) Induction of freezing tolerance, measured as electrolyte leakage, in one genotype grown under natural conditions. b) Hourly minimum temperature during the period when freezing tolerance is acquired. Temperature was measured ca. 300 m from the tree.

Gene expression during senescence.

Genes that is differentially expressed during senescence has been the main focus of many research projects for a long time, usually the senescence associated genes (SAG) have been studied and several broad studies have given interesting insights into this complex process (Lohman *et al.*, 1994; Buchanan-Wollaston *et al.*, 2003; Gepstein *et al.*, 2003; Buchanan-Wollaston *et al.*, 2005; Breeze *et al.*, 2011). The studies have shown that very quickly after senescence has been initiated the expression of catabolic genes increase greatly (Guo *et al.*, 2004). Most of the nitrogen that is to be recycled is located in the chloroplasts, therefore protein degradation within the chloroplast is followed by chlorophyll degradation. The most important SAG genes are supposed to be involved in these protein degradation events. Most research in SAG genes have been conducted in crop species, but the gene expression patterns during senescence in aspen seems by large to be similar (Bhalerao *et al.* 2003; Andersson *et al.* 2004).

Plant hormones and senescence.

Plant hormones are the plants main regulators and messaging system and are responsible for several vital growth and developmental processes in plants (Davies 1995; Aaron and Estelle 2009). Hormones are essential for responses to environmental cues that the immobile plants have to adjust to. Earlier studies on plant hormones were very focused on hormone application on macro scale and subsequent interpretation of the results (Ludwig-Müller and Lüthen 2015). Although they yielded initially interesting results, they could often not explain the effect in

any depth and many of these studies yielded little substantial evidence of the actual function of the hormones on a molecular level. Nowadays the field of plant hormones have developed, the number of known plant hormones has increased and their function have been more elucidated (see e.g. Vanstraelen and Benková 2012)

Senescence has been shown to be influenced by several plant hormones. The hormones have very different functions during the developmental stages of the plant (Davies 2010). Senescence promoting hormones are, in general, ethylene, abscisic acid, jasmonic acid and salicylic acid, these hormones tend to either promote or accelerate the senescence process. They could be produced as stress responses, ethylene has been shown to increase before the start of senescence (Aharoni and Liberman 1997). Cytokinin, gibberellic acid (GA) and auxins generally tend to have a delaying effect on leaf senescence (Gan 2008).

Abscisic acid (ABA) has been observed to decrease before senescence and has therefore been assumed to be associated with onset of senescence (Aharoni and Richmond 1978). If detached leaves are treated with ABA they show an accelerated senescence but the same experiment conducted on leaves still attached did not show the same result indicating a picture not as simple as ABA being a direct inducer of senescence, moreover it seems that the ABA has a strong connection to the metabolic state of the plant, specifically that of nitrogen and sugar that seems to affect the induction of ABA (Pourtau *et al.*, 2004; Buchanan-Wollaston *et al.*, 2005).

Jasmonic acid (JA) was shown to also have promoting effect on leaf senescence when detached leaves were given an external application of JA (Ueada and Kato 1980; Wasternack and Hause 2002). The response to application of JA to leaves have many similarities to the ABA treated leaves, although JA seems to have a stronger effect on leaves that are still attached compared to ABA. Senescing leaves in *Arabidopsis* have been shown to contain increased levels of JA in conjunction with increased biosynthesis and crosstalk with other hormones (He *et al.*, 2002; Sasaki *et al.*, 2001). Other studies have linked JA to decreased cytokinin levels however exactly how this connection is regulated or if it a general feature of senescence remains unclear (Ananieva 2004).

Salicylic acid (SA) has also been linked to senescing leaves (Morris *et al.*, 2000). SA seems to be more involved in promoting leaf senescence as it has been initiated and thus speeding it up as studies made by Morris *et al.*, 2000 have shown that lowered SA levels slowed the leaf senescence but didn't stop the initiation. The SA seems to be the hormone that makes the cells changes their state from senescing to the final cell death state (Morris *et al.*, 2000).

Ethylene is a hormone with many roles in the developmental process in plants. Ethylene has shown to be a strong accelerator of senescence and associated with leaf

abscission and fruit ripening (Zacarias and Reid, 1990). Ethylene seems to be induced in connection to biotic and abiotic stresses, and these increased ethylene levels can under certain circumstances initiate stress induced senescence (Ouaked *et al.*, 2003; Rieske *et al.*, 1995). The initiation of senescence by ethylene will only work if a certain age have been reached by the leaf (Jing 2005). Studies conducted by Kieber *et al.*, 1993 constitutive induced ethylene by making *ctr1* triple mutant didn't affect the timing of senescence. This is a strong indication for the age dependent regulation of ethylene, also several SAG genes are induced by ethylene (Grbic and Bleecker 1995). These studies of ethylene suggest that ethylene is a hormone with extensive crosstalk to other hormones also making it harder to clearly make a complete picture of the interactions of ethylene.

Auxins role during leaf senescence are so far contrasting and not very clear but recent investigations have shown that the role might extend into more areas than previously thought. Some experiments show that elevated auxin levels prior to senescence delays the process (Kim *et al.*, 2011). But this simplistic model has been investigated and a number of factors have come to light in the cell regulatory network among them many transcription factors seems to be regulating auxins, and in some plants the auxin levels increase during senescence further complicating the picture of auxins role during senescence (Aharoni *et al.*, 1979). Although most of this investigation into the role of auxins during senescence was performed in annuals and many food crops, if auxin functions similarly in trees remains to be seen.

Gibberellins are mostly associated with growth and seed development and germination (Sun and Gubler 2004).but their role in senescence was shown in early studies when leaf discs were treated with gibberellic acid, they tended to stay green much longer then untreated leaves (Fletcher and Osborne 1965; Beevers 1966). Gibberellins show a tendency to delay leaf senescence but the effect varies a lot between species and there is a huge difference in effect between the gibberellin species (Nooden 1998; Ranwala *et al.*, 1999). The delaying effect of senescence could also be considered that of a weak inhibitor, since the delay is often temporary and easily contracted by other factors (Back and Richmond 1971). Many studies where mutant plants with altered biosynthesis and perception of gibberellins did not have any clear effect on leaf senescence, some reports have shown delay but many confounded effects may be the cause rather than the gibberellic acid (Dill *et al.*, 2004; Richards *et al.*, 2001).

Cytokinins

Cytokinins have been considered as a main hormone for proliferation and are involved in many developmental processes such as nutrient mobilisation, shoot apical meristem activity, floral development, bud break, seed germination and – important for us - leaf senescence. The role of cytokinins in senescence has mainly been reported to be that of an inhibitor (Gan and Amasino 1995). If detached leaves of many plants are treated with cytokinin the leaf senescence will be delayed. A general decrease in levels of cytokinin and capacity to synthesize has been observed before leaf senescence is initiated in many plants (Gan S and Amasino 1997; Van Staden J *et al.*, 1998). In most plants the cytokinins are synthesized in the roots and then transported to the leaves, leaves can synthesize cytokinins as well but generally on a much smaller scale than the roots (Gan S and Amasino 1997).

The role of cytokinins in regulating the onset of senescence was tested by transforming tobacco plants (*Nicotiana tabacum*) with a chimeric gene, this gene had a senescence specific promoter driving the expression of the *IPT* gene (Gan and Amasino 1995). When senescence was initiated the *IPT* gene expression also started and the concentration of N-isopentenyladenine cytokinins increased. The increased concentration of cytokinins inhibited the onset of senescence in the tobacco plants but also blocked further expression of the *IPT* gene and overproduction of cytokinins, this indicated that the system was self-regulating and cytokinins could be considered a natural regulator of leaf senescence. Studies have indicated that cytokinins can function as an accelerator of senescence (Li *et al.*, 1992), so the role of the cytokinins during senescence is not as simple or clear as previously thought.

Biosynthesis of cytokinins

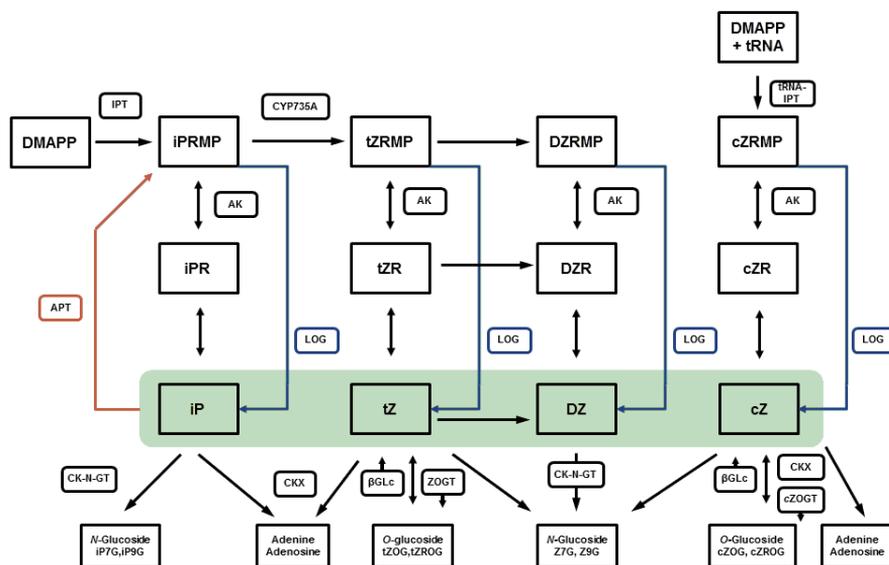


Figure 2: Cytokinin biosynthesis pathway for the four main cytokinins N^6 -isopentenyladenine (iP), *trans*-zeatin (tZ), dihydro-zeatin(DZ), *cis*-zeatin(cZ) (for other abbreviations, see Supplemental Table S1), and their two main activation pathways (MEP and MVA). Reaction pathways are indicated with arrows, with the names of the corresponding known genes written beside them. Reaction pathways shown to be reversible are marked with two-sided arrows (Sakakibara 2006).

The cytokinin family have a broad structural diversity as summarized in Figure 2. The distributions of the different cytokinins species are not universal and vary extensively from species to species and even during the developmental stage of the plant (Sakakibara 2006; Hirose *et al.*, 2008). The roles of the different cytokinin species is still being investigated and are not completely elucidated. The 4 main groups of cytokinins are N^6 -isopentenyladenine (iP), *trans*-Zeatins (tZ), *cis*-Zeatins (cZ) and dihydro-Zeatins (DHZ) a fifth group the aromatic cytokinins or topolins are present in some species. These cytokinins can be divided into 3 main categories, translocation form, bioactive form and storage and inactivated form. There are two pathways in the cytokinin biosynthesis, the dimethylallyldiphosphate pathway followed by the tRNA pathway, the tRNA pathway is poorly characterized and the actual infusion from tRNA is considered limited, it is believed that the cytokinins that are produced from this pathway are mainly *cis* type cytokinins (Murai 1994). The motivation for this assumption is mainly that the ratio of *cis* to *trans* tRNA is about 40:1 which would suggest that mainly *cis* type CK are derived from tRNA degradation (Vreman *et al.*, 1978; Klämbt 1992). The iP are generally considered as the dominant cytokinin with the highest affinity to receptors and therefore a high bioactivity (Schmitz and Skoog, 1972; Mok *et al.*, 1978). The *trans*-zeatins have

also been considered as a cytokinin species of high importance since they are often found in high concentrations in plants. The Zeatins have been implicated in *cis-trans* isomerization as first described by Bassil *et al.*, 1993. Several studies on the subject have been conducted where a weak conversion was shown (Suttle and Banowitz, 2000) but this is a debated subject since other reports have also detected no isomerisation (Kuroha *et al.*, 2002; Yonekura-Sakakibara *et al.*, 2004). The *cis* cytokinins were initially believed to only be a storage form and not have any bioactive function since they usually weren't present in large quantities and only showed weak activity in many species. But the plants where they appear in large quantities like chickpea (Emery *et al.*, 1998), maize (Veach YK *et al.*, 2003) and potatoes (Suttle *et al.*, 2000) special receptor (ZmHK1) have been detected that reacts to *cis* type cytokinins (Yonekura-Sakakibara *et al.*, 2004). Recent studies have shown that *cis* type cytokinin may be involved in completely different roles in plant development e.g. in stress responses (Gajdošová *et al.*, 2011; Schäfer *et al.*, 2015). The DHZ type cytokinins have mostly been ignored since they usually appear in very small concentrations or not at all in some plants. The DHZ seems to resist cytokinin oxidases much better (Armstrong 1994; Galuszka *et al.*, 2007), and it has been reported to be present in higher concentrations in seeds (Stirk *et al.*, 2012) suggesting that it might be utilized as a source for active cytokinins in germinating seeds.

The aromatic cytokinins (topolins) were the last to be discovered and have been shown to exist in some species like poplars and Arabidopsis (Strnad 1997; Tarkowska *et al.*, 2003). Some parts of the biosynthetic pathway seem to be shared with the other cytokinins, but a large part of it remains to be elucidated as with their function in plants (Mok MC *et al.*, 2005).

Cytokinin-related genes

The cytokinin related gene family in *Populus* contains some 85 genes, as listed by Immanen *et al.*, (2013) but about a further 30 genes including the PUP and ENT gene families are supposed to have a role in cytokinin metabolism and/or perception, with more being added as they are continuously being discovered or classified as cytokinin related genes

A Key enzyme in the CK biosynthesis is considered to be the IPT gene family (Kakimoto 2001; Takei *et al.*, 2001). They are regulating the first step in the biosynthesis of cytokinins by the dimethylallyldiphosphate (DMAPP) pathway or the tRNA pathway. The expression of *IPT* genes seems to depend on several factors, one of them being the status of inorganic macronutrients (Takei *et al.*, 2001).

Adenosine kinase (ADK) genes convert base-ribosides into riboside-monophosphate (Schoor *et al.*, 2011). The conversion back into the bio-inactive monophosphate seems to contribute heavily to the homeostasis within the plant. This system is a way for the plant to maintain a recycling of the cytokinins back into inactive form, plants where the ADK genes had been silenced an increase in the amount of adenine and adenosine could be observed as an effect of the lost recycling (Schoor *et al.*, 2011).

The cytokinin oxidases or dehydrogenases (CKX) cleave the active cytokinin compounds into the irreversibly inactivated degradation products adenine and isopentenyl aldehydes (Schmülling *et al.*, 2003). There are 8 CKX genes in poplars with gene structure well preserved also between species, the proteins share a very low homology with only one FAD-domain as a common feature (Immanen *et al.*, 2013).

Adenine phosphoribosyltransferase (APRT) convert cytokinin bases to the ribosidemonophosphate form and thereby inactivate the biological activity of the compound. In Arabidopsis 5 APT genes exist and the same number can be found in poplar as well but the studies of these genes in trees are very sparse. Studies where the APT1 activity was removed led to an increase in the concentration of cytokinin bases and induced several unwanted cytokinin related responses (Zhang *et al.*, 2013). The roles of APT2-APT5 in adenine metabolism have been questioned lately as they didn't show any activity with *in vitro* experiments although APT4-5 is detectable *in vivo* with enzymatic essays.

Lonely guy (LOG) activates cytokinins by converting the inactive nucleotides to the free base cytokinin directly. This direct activation pathway seems to play a vital role in the development of plants by regulating cytokinin activity directly. In populus 13 LOG genes can be found compared to eight in Arabidopsis, the *PtLOG5* gene has four orthologues', in Populus the function of this fourfold gene expansion have yet to be clarified (Immanen *et al.*, 2013). Increased expression of the LOG genes have shown to be connected to delayed senescence, In the LOG gene family, *LOG7* seems to be contributing most to the cytokinin conversion (Tokunaga *et al.*, 2012).

Conversion of the cytokinin riboside-monophosphates into base-ribosides is regulated by two adenosine kinase (ADK) genes. The genes seem to have a very big role in maintaining the purine nucleotide pools and regulation of plant growth and developmental processes (Moffatt *et al.*, 2000; Zrenner *et al.*, 2006).

In populus five histidine kinase (HK) receptor genes can be found (*CRE1a*, *CRE1b*, *HK2*, *HK3a*, *HKb*). The CRE/HK receptors are the main perceivers of cytokinins and increased expression of this gene family have shown to increase the responsiveness of cytokinins. The HK receptors initiate a phosphor relay system that in turn activates the response regulators that will trigger the output domain

associated with the specific response regulator (Kakimoto 2003). A high expression of the *HK3* gene has been associated with leaf longevity during senescence by activating the *RR2* gene, *HK3* and *CRE1* did not show any effect on senescence but instead seems to be linked to root and shoot development (Kim *et al.*, 2006; Ueguchi *et al.*, 2001).

Cytokinin response regulators (RR) consist of three main groups, types A, B and C. The RR is responsible for the final phase in the chain of signal transduction. There are eleven type-A RR genes in populus, six type-B RRs and eight type-C RR in total 25 genes in the RR gene family. The A-type RR are thought to mainly act as negative regulators and type-B as positive regulators of cytokinin signalling (Kakimoto 2003). The type-C RR seems to very specific in where they are expressed since they have only been found in pollen grains and germinating seeds (Horák *et al.*, 2008, Gattolin *et al.*, 2006). The knowledge if the function of the type-C RR in populus are still very sparse since they have only recently been discovered and they might have other functions in the populus species then what have already been reported for type-C RRs.

The histidine containing phosphotransmitters (HP) family contains 14 genes in *populus*, they are responsible for the translocation of the phosphor group from the *CRE1/HK* receptors to the response regulators located in the nucleus (Punwani *et al.*, 2010). The *AHP6* or *PtHP*-like gene acts like a pseudo gene and gives an inhibitory role on the phosphorelay (Mähönen *et al.*, 2006). The HP-like genes function is still unknown but it has been shown that where it is expressed it creates a domain of very low cytokinin signalling and therefore lowering the activity of cytokinin effects. In populus the *AHP4* genes has four orthologues that like the *PtHP*-like genes have lost the His residue that are vital for the phosphor accepting function of the protein. This might indicate that these genes might also act as negative regulators of cytokinin signalling (Hutchison *et al.*, 2006; Chu *et al.*, 2011).

The cytokinin response factors (CRF) contain eight known genes in the CRF transcription factor family in *Arabidopsis* and four of them could also be found in *populus* but investigation and reliable data in *populus* is severely lacking. Recent studies have also indicated that the number of CRF genes is even higher than previously thought (Rashotte *et al.*, 2010). The cytokinins CRF role have shown to be involved with leaf development (Brenner *et al.*, 2012).

Carbohydrates and senescence

The synthesis of carbohydrates by photosynthesis throughout the summer is a vital process that will ensure the survival of the tree during the winter months. In the northern forest summers are short and therefore the time for carbon allocation is limited. The tree collects carbon in addition to the annual growth to be able to

prepare for the winter, and the formation of buds which is the guarantee for next year's growth. Late in the season the carbon allocation is shifted to below ground to roots and mycorrhizal fungi (Lippu 1994; Kagawa *et al.*, 2006). Timing of autumn senescence is therefore critical, entering senescence too early will make the trees not gather enough carbohydrate stores for adequate winter survival or protection for the buds from extreme cold. That carbohydrates play a role in senescence in many plants is clear as many studies indicate that increases in sugar concentration act to initiate leaf senescence (Wingler *et al.*, 2009; Wingler and Roitsch 2008), however whether low or high sugar status initiate/delay senescence is still a conflicting subject since evidence supports both scenarios (Hensel *et al.*, 1993; Quirino *et al.*, 2000; Masclaux 2000; Stessman *et al.*, 2002; Nemeth *et al.*, 1998).

The function of anthocyanins in leaves is most likely photoprotective which was shown by the studies conducted by Gould *et al.*, (2004) and Hoch *et al.*, (2003). The details about the actual function of anthocyanins have been heavily debated, and anthocyanins are also produced when the young leaves develop and start to green or when stressed by biotic or abiotic factors (Field *et al.*, 2001; Steyn *et al.*, 2002). A common theme for anthocyanin induction is that they are produced when the plant is in a vulnerable state or experiences stress. Many plants can accumulate anthocyanins during senescence which can be considered as a vulnerable state for the leaf when many protective compounds and dangerous substances are degraded or moved within the cells (Wheldale 1916; Sanger 1971; Chang *et al.*, 1989; King 1997; Kozłowski and Pallardy 1997; Field *et al.*, 2001). Carbohydrate status has also been linked to anthocyanin production in several studies (Weiss *et al.*, 2000; Larronde *et al.*, 1998; Hara *et al.*, 2003). When the plant is exposed to high concentrations of sugars this triggers the *MYB75/PAP1* gene for biosynthesis of anthocyanins (Teng *et al.*, 2005; Solfanelli *et al.*, 2006). However even with multiple studies showing various functions for anthocyanins it might be that it is only a way to spend the photosynthates that have been accumulated in great quantity, and as such the anthocyanins might not have a function at all in some cases.

Girdling as an artificial carbohydrate concentration shifter

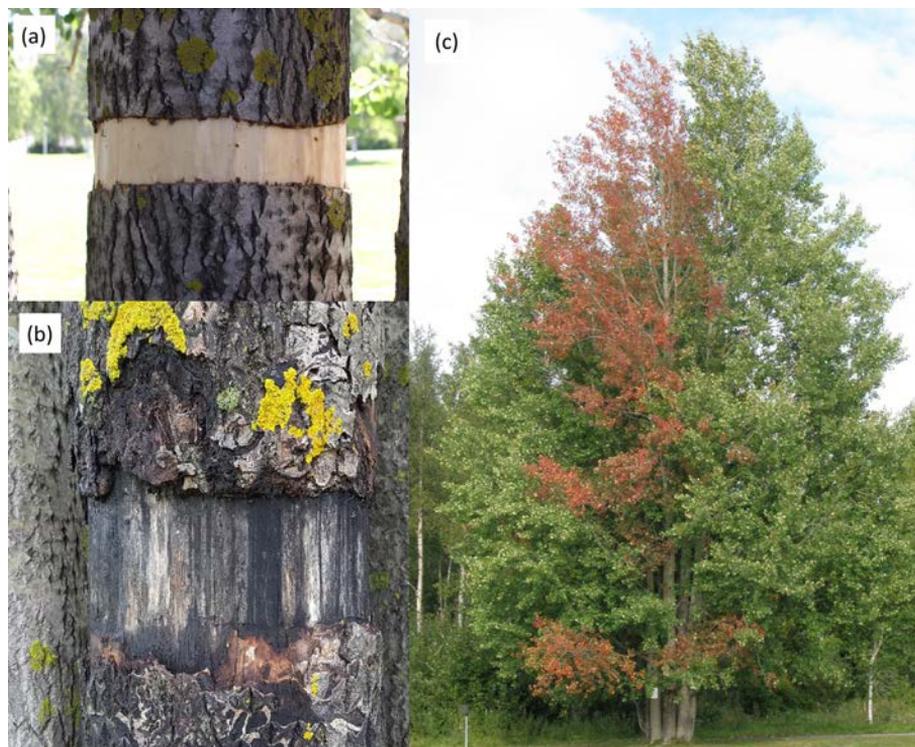


Figure 3: a) A newly girdled aspen stem b) the same stem 10 years later c) Our “favourite free growing aspen” designated as tree 201 with the girdled stem showing anthocyanin accumulation.

Girdling is a technique where the outer bark of a tree is removed, including the phloem but leave the xylem intact (Regier *et al.*, 2010). Girdling will induce a block in transport down to the roots via the phloem, but root to canopy transport via xylem is still functional. In most trees this will mean that the roots will starve to death and the tree will subsequently die but this is often not the case in aspen trees (*Figure 3*). Aspens are often connected to each other by the root system since most aspens propagate with root suckers and the root connections are often retained even though the trees grow to maturity. The root system and its connection to close by trees can therefore be quite extensive and the roots will still be supplied by its neighbour's stems with nutrients. When a tree is girdled a build-up of carbohydrates will start in the canopy, since a lot more could be produced than can be consumed or utilized with the root sink cut off. This will lead to an artificial carbohydrate overload in the canopy of the tree.

Nitrogen and the effects of senescence

Trees growing in the northern latitudes not only have to endure the long winters but they grow on very nitrogen poor soils (Tamm 1991; Vitousek and Howarth 1991). The nitrogen that exist in the soils tend to be in organic forms that are bound in complexes that the trees can't utilize (Lipson and Näsholm 2001; Vitousek *et al.*, 2002). The breakdown of the nitrogen rich compound by bacteria or time are very complex and time consuming and usually will generate much less nitrogen then the trees will need (Schimel and Bennett 2004). This constant lack of nitrogen means that nitrogen will be the main limitation of growth for the trees, so for increased productivity in these forest system additions of fertilizer is a common practice (Hyvonen *et al.*, 2008; DeVries *et al.*, 2009). It is common knowledge that you should not fertilize your deciduous plants in late summer or autumn since this can compromise their winter survivability (Sigurdsson, 2001). Even though this has been known for quite some time it is much less so understood as a biological function. Recently some experiments have started to illuminate what is actually happening in the plants when additional nitrogen is added late in the season but mostly in annual plants (Gutiérrez-Boem *et al.*, 2004; Blandino *et al.*, 2015).

Arginine vs. conventional fertilizers

The majority of fertilizers in commercial use consist of inorganic nitrogen such as potassium nitrate (KNO_3), ammonium (NH_4NO_3) or urea ($\text{CO}(\text{NH}_2)_2$) these inorganic fertilizers although effective on regular crops tend to have some adverse effect on deciduous plant species such as decreased winter survival in northern climates by extending the growth period of the roots and thus compromising their timing of acquiring winter dormancy (Johnsson *et al.*, 2000) but if trees grow in less harsher climates late fertilisation can increase the winter survivability (Andivia *et al.*, 2012). This shows that a number of different environmental factors must be considered to fully understand the effect of nitrogen addition. Trees that grow for a very long time and usually don't have the luxury of a limitless supply of nitrogen in the soil and what becomes available is through a slow trickle when old organic materials decompose and becomes accessible organic nitrogen (Schulten *et al.*, 1998; Yu *et al.*, 2002; Andersson *et al.*, 2005). Deciduous trees that are growing on soils poor in nitrogen have shown to reabsorb a higher percentage of the available nitrogen in the leaves during senescence compared to other trees (Rennenberg *et al.*, 1998; Lovett *et al.*, 2004; Chapman *et al.*, 2006). But the decomposition of litter and other organic tissue result in various organic nitrogen rich compounds. Experiments have shown that some trees have a higher uptake capacity for organic nitrogen sources then inorganic, this makes sense since the trees would be evolutionary geared towards the organic nitrogen that would be the main source for the most of the trees lifespan. This doesn't necessarily mean that they grow better on organic nitrogen. Recent studies (see e.g. Näsholm *et al.*, 2009) have shown that also

inorganic nitrogen is being utilized by trees although it is still much to learn about how big the inorganic vs. organic contribution to the N status of the tree is. Organic nitrogen is available in many forms, free amino acids, peptides and protein bound amino acids. Studies have shown that more than half of the organic nitrogen in soils is composed of protein bound amino acids and peptides (Senwo *et al.*, 1998). The trees will need specific transporters for each nitrogen form to be able to utilize it, this creates problems for the trees when it comes to the many forms of the organic bound nitrogen, fortunately mycorrhizal fungi can absorb the organic nitrogen by degrading polymeric nitrogen and provides the tree with nitrogen in exchange for carbohydrates and thus giving the tree access to these otherwise unobtainable nitrogen forms (Smith & Read 2007). These discoveries have led to the development of new fertilizers (arGrow®) that are based on amino acids such as nitrogen rich arginine. The organic fertilizers have shown much better uptake in conifers compared to conventional fertilizers. Arginine is also the compound that trees use for storing excess nitrogen and it is a major transport molecule of plants (Nordin *et al.*, 1997; Bausenwein *et al.*, 2001; Rennenberg *et al.*, 2010).

Aspens and the measurements of autumn senescence.

Aspens as model system

The genus *Populus* belongs to the *Salicaceae* family, and contains some 35 different species. The *Populus* species and their hybrids are spread out over a wide geographical area concentrated on the northern hemisphere. Furthermore *Populus trichocarpa* was the first tree that had its genome fully sequenced (Tuskan *et al.*, 2006). This provides a very good platform for genetic and molecular studies for the *Populus* species since an abundance of information is available.

European aspen (*Populus tremula*) (Figure 4) is native to most parts of Europe and continues to stretch all over Russia to China, in Sweden they are found throughout the country (Cadullo and de Rigo 2016). Aspens are not regarded as a high value commercial tree since it has a rather soft wood compared to other trees, although the tree is well suited for biomass crop since it grows very rapidly (Savill 2013, Mackenzie 2010). Aspen is a pioneer species that tend to populate areas that have been emptied by forest fires or clearcuttings (Latva-Karjanmaa *et al.*, 2007). This ability to grow fast in the juvenile phase and out-compete other trees usually locates aspens at the edge of fields or roadsides where they often cluster in clonal stands. The European aspen has been chosen as the model species for autumn senescence in deciduous trees. The hybrid aspen clone T89 (*Populus tremula* x *tremuloides*) is used commonly in the lab, and provides an abundance of tools and information needed for the study of senescence in aspens.



Figure 4: Free growing aspen” (*Populus tremula*) in the Umeå region, the photos were taken on September 19.

The *Populus tremula* was used as model in all three papers presented in this thesis. The T89 clone has turned out not to be a suitable system for our senescence studies, neither in the lab, nor in the field in our climate it obviously doesn't senesce like our “normal aspens” do. This “poor behaviour” of T89 during senescence is most likely caused by poor adaptation to our climate. One particular aspen that is growing at the Umeå university campus have been the focus of many studies on aspens and autumn senescence (Keskitalo *et al.*, 2005; Fracheboud *et al.*, 2009; Bhalerao *et al.*, 2003; Andersson *et al.*, 2004) and also studied in Paper I and Paper III. The aspen is designated 201 (Figure 3c). It has in fact recently appeared in two plant physiology textbooks as a representative for autumn senescence in trees. Recently the genome of the 201 was sequenced and is now publicly available as the first genome assembly of *Populus tremula* (*P.tremula* V1.1 www.popgenie.org).

SwAsp garden and free growing aspens

Selecting aspens candidates for the experiments was mainly conducted by using the already abundant aspen stands that grows throughout the region in Umeå. Usually free growing aspens tend to cluster since established trees will continue to propagate

through root suckers in extension to seed propagation. The aspen trees ability to form clusters by root sucker propagation will make the likelihood of a cluster to be clones very likely. The aspen stands can be easily checked by a few select markers through QPCR to ascertain if they are clones, the trees used in Paper I and the field fertilisation experiment in Paper II was checked with microsatellite markers to confirm that they were clonal, all grouping within a stand in Paper I and Paper II was shown to be of clonal origin.

The Swedish aspen collection (SwAsp) garden was established in 2004 and contains aspens collected from 12 locations in Sweden covering most of the country from the latitudinal span of 56.3°N to 66.2°N.(Luquez *et al.*, 2008; Ingvarsson *et al.*, 2008). Two gardens have been established and contain the same collection of trees, one northern site is in Sävar (63.4°N) close to Umeå and the other in Ekebo (55.9°N) that is in the southern Sweden. The gardens contain 12 clone groups with 10 clones in each group. The clone groups have been collected in a manner so that it will cover a wide geographical area of Sweden to maximize the phenotypical variation.

Identifying onset of senescence.

To be able to measure autumn senescence on a macro scale we use the visual cue of chlorophyll breakdown. The breakdown of chlorophyll is strongly linked to the progress of senescence and using this reference can tell us how far the senescence has progressed (Ougham *et al.*, 2008). The CCM-200 (Opti-sciences, Hudson USA) was used for all chlorophyll measurements presented in this study, the CCM200 is a non-invasive hand held spectrophotometer that measures a relative chlorophyll content value. The relative chlorophyll content values have been shown to correlate very well with actual chlorophyll concentrations in the leaf (Richardson *et al.*, 2002). Although “onset of autumn senescence” in theory is simple, to define it out of experimental data is not straightforward. There is a constant “tear and wear” degradation of chlorophyll, but this degradation is typically much accelerated, in our climate typically in mid-September. Although it is not obviously whether there really is a “date of onset of senescence” or a gradual change in chlorophyll degradation speed that happen over a few days, we need a way to translate the curves for rather noisy measured chlorophyll levels to dates, in order to compare between treatments.

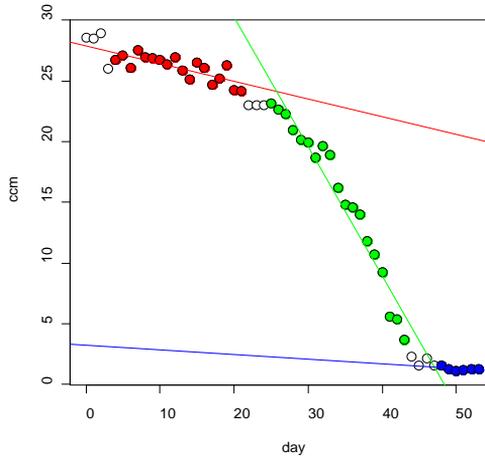


Figure 5: Chlorophyll values and the onset day are calculated by the intersection of the red and green line, the end of senescence is calculated by the intersection of the green and blue line.

A method was constructed using an R script (R Development Core Team) where a proxy of the onset of senescence was calculated based on the chlorophyll degradation curves (Figure 5). We have found that the nitrogen content of an aspen leaf and the relative chlorophyll content value obtained by the CCM-200 correlated linearly as shown in Figure 6.

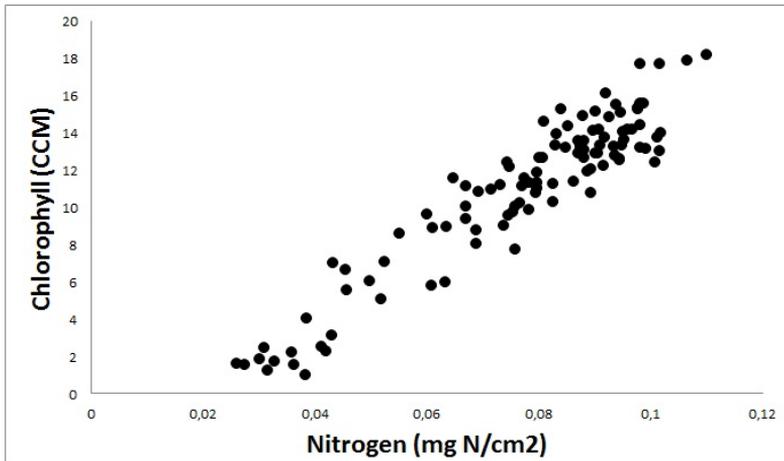


Figure 6: Nitrogen content plotted against the relative chlorophyll content value (CCM).

Objectives

The objectives of this thesis are to understand the molecular regulatory factors governing the actual onset event of autumn senescence in European aspen. The main factors that have been investigated are:

- The effect of increased levels of carbohydrates studied by girdling.
- The effect of organic and inorganic nitrogen studied by “precision fertilisation”.
- The effects of cytokinins studied by profiling of cytokinins species and gene expression..

Results and discussion

Carbohydrate accumulation induce premature senescence in aspens (Paper I)

Girdling in aspen trees induced a number of different effects: 1) a premature senescence 2) anthocyanins levels are either maintained or increased after onset of senescence 3) flowering 4) slowed down senescence progress compared to “naturally induced” senescence. The increased concentration of photosynthates has obviously a profound effect on the trees capacity to initiate senescence as shown in Paper I. Initiating senescence later will increase the annual photosynthetic yield for the tree, so there exist a trade-off between late initiated senescence and risking frost damage that will cause less nutrients to be remobilized. The girdling study showed that an increase in carbohydrates gives an earlier induction of senescence and an increase in anthocyanin. Onset of senescence was, in our case, about 2 weeks earlier than control trees. The time it takes to complete senescence in the girdled trees was on the other hand doubled and the rate of degradation in chlorophyll was therefore only half the usual rate. The slower rate of senescence could perhaps be due to the weather conditions being more favourable earlier in the season as senescence are conducted at a slower pace in warmer climate (Fracheboud *et al.*, 2009), or some other “internal factor” in the tree could be responsible for the change. The “competence to senesce” for our aspen trees seems to develop around 20 days after bud set. The trees that were girdled had set buds roughly in the last week of July and started showing senescence symptom around august 20. Maybe initiation of senescence in the girdled trees would be even earlier if not for the inability to start until bud set, this would be in line with the theory that bud set in aspens is a regulative ARC.

The maintained or increased anthocyanin levels that were linked to the onset of senescence in girdled trees can be a way for the tree to increase the protection from photo-oxidative stress (Schaberg *et al.*, 2008; Field *et al.*, 2001). The extra protection of the anthocyanin could help the tree to more efficiently extract nutrient from the leaves, which correlates with our data that indicates a higher remobilisation in girdled trees. The extra carbohydrates that the tree has accumulated are very likely used for the synthesis of the anthocyanin at the onset of senescence. It should be noted that in many other species it has been observed that increased sugars concentration alone can induce anthocyanin biosynthesis (Weiss *et al.*, 2000, Larronde *et al.*, 1998, Hara *et al.*, 2003) but in aspens it seems the sugar-induced pathway is not initiated until leaf senescence is has begun, as we rarely have observed anthocyanin accumulation in green leaves.

The variation in onset of senescence in different aspen clones indicates that a different selection pressure is in effect that seems to be linked with the nutrient status of the soils. This effect was observed in the girdling experiments where the start of senescence was delayed in one stand with chlorophyll levels that was much higher than in trees growing under normal conditions, as the chlorophyll levels are related to nitrogen levels in the tree.

Inorganic nitrogen delays senescence but organic nitrogen do not affect senescence (Paper II)

The addition of small amounts of nitrate during autumn gradually slows the period it takes to complete the senescence, counted from initiation to leaf abscission. The effects are subtle at small amounts of nitrate but as the amount increases a threshold seems to be crossed where the plant almost puts the senescence progression on hold and a shift in the metabolic process can be observed. For our “full-grown aspens” these changes could be observed in as small amount as 4g of nitrate added during a growing season. As mentioned earlier alders (*Alnus spp.*) do not go through autumn senescence. Alders can do this and thrive without the remobilized nutrients because of the symbiosis with nitrogen fixing filamentous bacteria (*Frankia*) that grows in the root nodules of the tree. The tree effectively gets the nitrogen it needs and can spend more time to collecting additional carbohydrates for the oncoming winter. This extra time of carbon allocation is obviously outweighing the loss of nitrogen and other nutrients (Hoch *et al.*, 2003; Fajardo *et al.*, 2013; Vanderklein and Reich 1999). As the growing season in our climate is around four months, a couple of weeks – as we observe in Paper II - can make a significant difference at our latitudes.

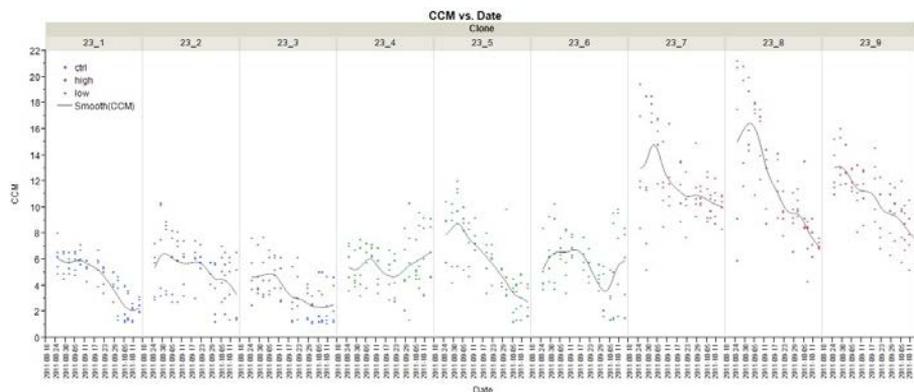


Figure 7: Nitrogen treatment of aspen tree growing outside. The trees were treated with no (ctr, left), Low (0,14g, middle) and high (1,38g, right) amounts of nitrogen every biweekly from July until mid-August.

The nitrogen addition experiments in Paper II established that fertilized aspens could acquire an altered senescence profile. When higher amounts of nitrate were applied the onset of senescence was shifted, up to 2-3 weeks. The trees that experienced a more profound delay also conducted senescence faster, the cause was most likely that of temperature shift, which in the beginning of October reaches down to freezing during the nights. The trees in the small common garden experiment in Paper II was treated with a high dose of nitrogen, they showed no onset of senescence at the “usual onset time”. In another common garden experiment the results from one clone group can be seen in *Figure 7*, the trees was treated with no (Ctr), Low (0,14g) and high (1,38g) amounts of nitrogen biweekly from July until mid-august. The experiment showed that trees supplied with a high dosage of nitrogen have twice the amount of chlorophyll and even though the senescence is conducted with the same rate or higher as non-treated trees they don't reach half their initial chlorophyll value before frost killed the leaves.

When trees were exposed to two different regimes of nitrogen (Paper II), one with only inorganic nitrogen (KNO_3) and the other with organic nitrogen (arginine: arGrow®) through an IV-bag infusion treatment (*Figure 8*) they expressed different senescence profiles. Senescence could be delayed already at the lowest supplied nitrate dose (4 g(4g). However, increased dosage of up to 60g of nitrate didn't make much additional difference, hence there may be a threshold effect by nitrate fertilization on senescence. The senescence profile for the arginine treated trees showed no significant difference from control trees in any supplied concentration. A metabolic profiling revealed a clear difference in the metabolome between the two treatments. Aspens that received nitrate seemed to acquire an altered metabolic profile in a dose dependent manner (Paper II, *Figure 7*).



Figure 8. Aspens with IV-bags attached, used for the nitrogen infusion in Paper II.

Cytokinins and senescence.

The tree used for the cytokinin profiling in paper III is our “favourite” 201 where timing of onset of senescence have been studied for several years before (Keskitalo *et al.*, 2005; Fracheboud 2009). These studies have shown a very stable date of onset of senescence from year to year indicating a rigid system for the initiation of senescence.

The extensive profiling of cytokinins in Paper III provided insights into the cytokinin hormonal status during the autumn. Most previous studies have focused on herbs/crops (Raspor *et al.*, 2012, Mornya *et al.*, 2011), but there are also studies performed in conifers (Rasmussen *et al.*, 2009) with the focus on growth and development, obviously not autumn senescence as an evergreen do not undergo autumn senescence. Most studies in annuals have reported that senescence is usually preceded by a decrease in cytokinins (Gan and Amasino 1995; McCabe *et al.*, 2001; Ori *et al.*, 1999). Some authors have also argued that the decreasing cytokinin levels are the cause for initiation of senescence (Hirose *et al.*, 2008; Kudo *et al.*, 2010; Singh *et al.*, 1992).

The cytokinin profiles generated in Paper III spanned over 3 consecutive autumns. Senescence was initiated on approximately the same date (within a few days), like in earlier studies on this particular aspen (Fracheboud 2009, Keskitalo *et al.*, 2005). Our measurement spanned from end of July to beginning of October with over 30 time points collected each.

The active cytokinin levels of IP decreased slight during the first half of the autumn in the year 2012-13 whereas in 2011 the IP had a more or less on a constant abundance throughout the season. Although the levels were somewhat inconsistent in 2012 (*Figure 9*), a small increase of IP levels in the last week was observed 2012-13. Since IPs and tZs are considered to be the main active cytokinins this profile would suggest that IPs, the most abundant active cytokinin species in our samples, is unlikely to block or delay senescence. The *trans*-zeatins exhibited a somewhat similar pattern to the IPs but decrease until late stages of senescence when they actually started to accumulate again. In 2011, both IPs and tZ leaves were more or less stable throughout the season. *cis*-zeatins had a very unclear pattern, all years looked different except for a strong increase in last stages of senescence that was consistent. *cis*-Zeatins were also much less abundant (concentrations ranging from 0,2-0,6 pmol/g). The dihydrozeatins which are considered to be the CK with least activity were in our profile also the least abundant of all the CKs (ranging from 0,05-0,1pmol/g). The DHZ behaved similar over years, but did appear in a twofold higher concentration in 2012 and 2013 compared to 2011, and an increase in concentration in late stages of senescence was also observed for DHZ. This late

season increase in *cis*-zeatin may be linked to increased stress in the leaves; recent studies have indicated that the *cis*-zeatin may play a role in stress responses (Schäfer *et al.*, 2015).

In aspens the cytokinins seemed however to fluctuate much less and only a small increase in the active cytokinins was observed (Paper III: sup figure 4). Therefore we do not believe that diurnal fluctuations in CK levels can explain any of our results.

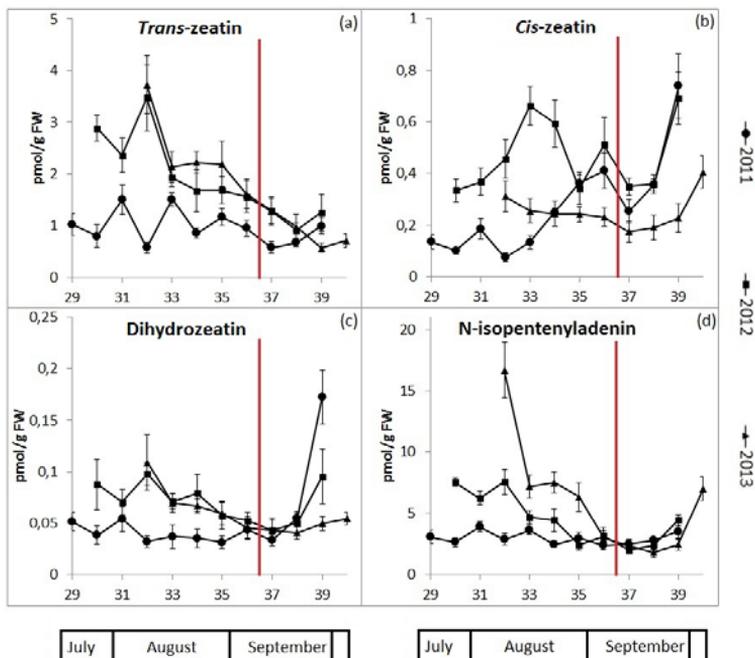


Figure 9: Concentration of active cytokinins of four isoprenoid CK groups, a) *trans*-zeatin, b) *cis*-zeatin, c) dihydrozeatin and d) N-isopentenyladenine, in triplicate samples of aspen leaves, presented as the sum of ribosides and bases. The values (in pmol per g fresh weight leaf material) are averaged for each week of measurements over a period of 11 weeks in total (weeks 29-40). Error bars show standard deviation (\pm SD). The approximate date of onset of senescence is marked with a red vertical line; September 10 occurred in week 37 in 2012-13 and in week 36 in 2011.

Studies by Novakova *et al.*, 2005 have shown that cytokinins can fluctuate in levels during the day, a fourfold change in concentrations of CKs over the period of one day is not uncommon. In aspens the cytokinins seems to fluctuate much less and only a small increase in the active cytokinins was observed (Paper III:sup figure 4).

Cytokinins also have the capacity to interconvert between the different subgroup types and from the active to inactive forms (Sakakibara 2006), that's why a view of the total pool of cytokinins might give a larger view of cytokinins during autumn senescence. The total pools of cytokinins were showed large variations from year to year (Figure 10e). The pool of iP type cytokinins (Figure 10d) generally decreased before onset of senescence and then remained somewhat steady. *trans*-Zeatin pools (Figure 10a) being the most abundant cytokinin species displayed relatively constant levels over 2 years but in (2012), the pool size showed a gradual increase to twofold levels over 2011 and 2013. *cis*-Zeatin pools (Figure 10b) showed a gradual increase during the whole season but with a variation in concentration between the years. Dihydrozeatin (Figure 10c), which surprisingly was the second most abundant cytokinin type if all, not only active, forms were considered showed a rather constant concentration in 2011 and a steady increased in 2013 and only an initial increase in 2012 followed by steady levels until the end of senescence.

The knowledge of aromatic CK, topolins, and their function is still very fragmented, and as they do not appear in every species their function may be very specialized. In aspen topolins are present they were therefore also investigated for any potential interesting profile during autumn senescence. Their profile showed an unclear picture (Paper III, Fig5), in 2 years they kept the same level throughout the season and a very weak increase could be observed after initiation of autumn senescence. In one of the years (2012) however, a strong increase was observed already in the beginning of August and kept at this high level until just before onset of senescence when levels decreased to initial levels. The very different yearly profiles and a seemingly constant baseline concentration suggest that topolins are most not involved in regulating autumn senescence.

We also profiled expression of genes related to cytokinin biosynthesis, regulation and signalling. RNA-seq data from one year (2011) showed that out of the 85 previously identified "cytokinin associated genes", expression of 32 was found in leaves. We also profiled 22 additional genes that may have a role in cytokinin perception (PUP, ENT, AK, CRF and AP2/ERF). Generally the expression profiles for CK metabolism genes could not obviously be connected to the levels of the corresponding metabolite (Paper III: Fig 6). Some genes like HK3a and HK3b showed a strong increase in expression before the onset of senescence and well after the initiation event. Since HK3 is a receptor gene responsible for cytokinin signalling and sensitivity, this expression profile of *HK3* would correspond to an increased responsiveness to cytokinins just before the onset of senescence, which

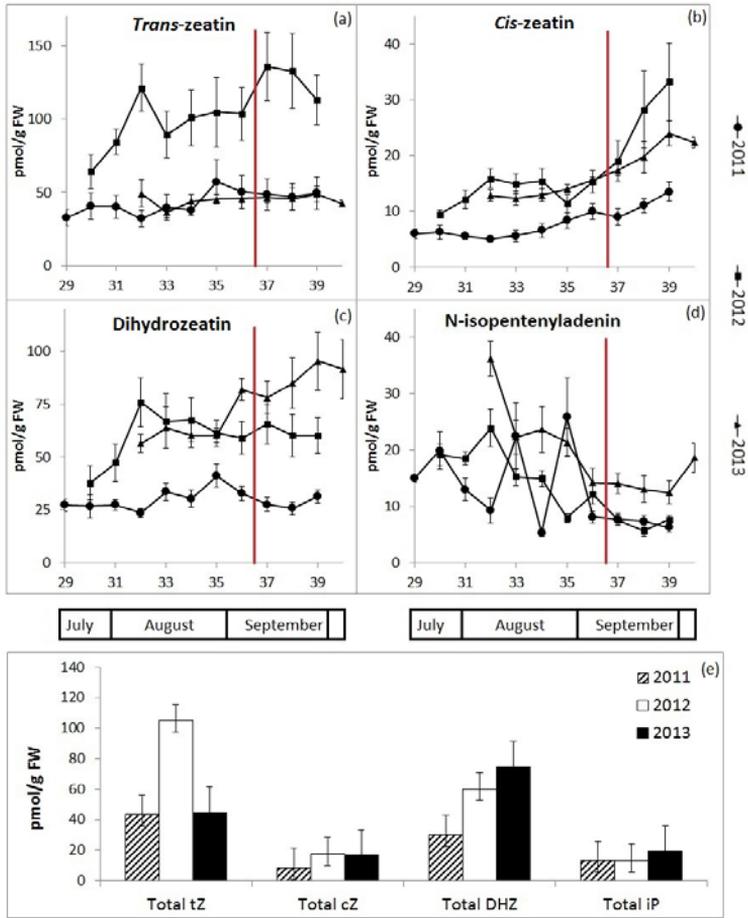


Figure 10: Total concentration of cytokinins in four isoprenoid CK groups, a) *trans*-zeatin, b) *cis*-zeatin, c) dihydrozeatin and d) N-isopentenyladenine, in triplicate samples of aspen leaves, presented as the sum of CK nucleotides, ribosides, bases and *N*-*O*-glucosides. The values (in pmol per g fresh weight leaf material) are averaged for each week of measurements over a period of 11 weeks in total (weeks 29-40). Error bars show standard deviation (\pm SD). The approximate date of onset of senescence is marked with a red vertical line; September 10 occurred in week 37 in 2012-13 and in week 36 in 2011. Subfigure e) depicts the average total amount of cytokinins over each year.

would be opposite to what would be expected if one would assume that decreased levels of cytokinin receptors could explain why aspen leaves start senescence in the autumn.

Considering the results from paper III together with earlier studies in aspen, a decrease in the concentration in one of the CK types is unlikely to explain the well timed and regulated onset of autumn senescence in aspens.

Conclusions and future perspective

Although all three papers of this thesis deal with factors initiating autumn senescence in aspen, it is not obvious how they all are connected. One possibility is that there exists a nitrogen/carbohydrate switch upstream from the daylight trigger that can initiate senescence and the increased carbohydrate status also triggers anthocyanin production but only after onset of senescence

The results of Paper II indicated that when the tree receives enough nitrogen the completion of senescence is given less priority or actively delayed by the tree to prolong the carbon assimilation at the cost of decreased nitrogen remobilisation from leaves. The mature aspens used in the organic/inorganic nitrogen experiment in Paper II didn't react to treatment as dramatically as the juvenile aspens in the first nitrogen addition. The more mature trees may handle the extra supplied nitrogen differently since they have more total biomass to distribute the received nitrogen and they also have the root connection to neighbouring aspens, this might allocate the nitrogen more evenly than trees grown without root connections. But another obvious explanation is that the dose they were giving was much lower, the young trees in the first experiment in Paper II had two-fold increased chlorophyll levels (that seems to mean double the amount of leaf nitrogen) while the older trees did not get enough nitrogen to change chlorophyll levels. There apparently seems to be a threshold for nitrate sensing that the tree perceives and accordingly changes senescence, but if trees get overloaded by high amounts of nitrate big effects on chlorophyll levels and senescence occurs. A question that needs to be addressed is if the observed metabolic shift was caused by decreased chlorophyll or if the decreased chlorophyll was the reason for the shift in the metabolome. Another issue that maybe should be better studied is if the "shift" that was observed is an effect of how we calculate the onset. Our current model for calculating the onset of senescence (Paper II) was developed in trees not highly fertilized and calculates a break between two lines in the chlorophyll concentration curve (*Figure 5*) which is less obvious in the trees in a high nitrogen regime (Paper II Fig1). Maybe measurements on more leaves and more often would give less noise and better precision in the calculations.

The data from paper III showed that the pattern of the isoprenoid cytokinins during autumn senescence is unlikely to explain onset of autumn senescence in aspens. The senescence trigger in aspens and other trees that initiate senescence by the calendar could potentially have a separate signalling transduction chain and that this does not include CK. However if the CK are acting downstream of the trigger the aspens ability to acquire competence to senescence that could be linked to decreased cytokinin levels which would give cytokinins the role of blocking early induction of senescence instead of promoting senescence by decreased concentration, this would mean that increased CK levels through external application would still cause

delayed senescence but not hinder the trees from initiating senescence, although further studies is required to elucidate this.

The observed delay in both initiation and progression of senescence in Paper II could be due to increased levels of cytokinins and measuring CK during senescence in trees receiving inorganic nitrogen would confirm if the role of cytokinin could also extend to a speed regulator of senescence in aspens.

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