2.3. Plant pigments and antioxidants (terpenoids) as protectors against low temperature stress

Terpenoids, including the carotenoid pigments, chlorophylls, as well as tocopherols and quinones (plastoquinones) are amongst the main and essential components of every thylakoid membrane. In a wider sense, including the species outside the plant kingdom, biological membranes without terpenoids do not exist in practice (Ourisson et al., 1994).

Thylakoid membranes are exceptionally sensitive and thermolabile, compared to other biological membranes. In many literature data the important significance of tocopherols together with zeaxanthin in stabilising and protecting the membranes from oxidative damages is considered. (Tardy and Havaux, 1997). Some authors attribute to tocopherol the effect, similar to the effect of cholesterol on the biomembranes (DellaPena, 2003).

Carotenoid pigments

The carotenoid pigments, being an important part of the chemical composition of almost every plant, and many other organisms as well, have endogenous content and metabolism, which is exceptionally constant in higher plants (Bungard et al., 1997). The main two functions of carotenoids in plants are related to the light phase of the photosynthesis and the protection against photodamage (Holt et al., 2005). They act as light-harvesting pigments that are the first to capture the light energy and, consequently aim it to the reaction centre of both photosystems by the chlorophylls *a* and *b*. Furthermore, they act as photoprotectors, filtering the short-wave radiation, quenching the triplet chlorophyll forms, neutralising the ROS that were formed (Li et al., 2009; Triantaphylides and Havaux, 2009). Thanks to their specific structure they participate in sustaining the physico-chemical properties of biomembranes in stress conditions and protect them against oxidation (Woodall et al., 1997; Gruszecki, 1999, 2004). The main and most important chemical property of carotenoids is their pigment property for absorption of light with a certain wavelength, due to their system of multiple (over 9) conjugated double bonds (Gruszecki and Srzalka, 2005).

The two most important carotenoids, present in most of the plants are the lutein and β-carotene. β-carotene has orange-red colour. It has non-polar molecule, containing two β-rings on both sides of the molecule. Like all carotenoids (and xanthophyll) molecules, β-carotene is also a tetraterpenoid, which means its molecule contains 40 carbon atoms. This is so, because biosynthetic pathway of carotenoids begins from 8 molecules of isoprene binding together, and forming two

molecules of geranylgeranyl pyrophosphate (van Arnum, 1998). After that lycopene is produced, starting the metabolism of carotenoids and xanthophylls. Being a non-polar molecule, β-carotene is located inside plant membranes and contributes to their fluidity (Strzalka and Gruszecki, 1994). Therefore, its spatial arrangement inside membranes is not so well-defined, like the xanthophylls for example, but it can form different angles with the surface of the membrane (Van de Ven et al., 1984; Gruszecki, 2004).

The lutein is present in a significant amount in higher plants (Johnson et al., 1993; Bungard et al., 1997), whereas in some of them (including *Arabidopsis*) has the highest content amongst the carotenoid pigments (Havaux et al., 2007). According to the same authors its content is between 30-60% from the total content of the carotenoid pigments in higher plants. Carotenoid pigments are tightly related to photosynthesis and most of them are exceptionally important in the formation and function of the antenna complexes (Standfuss et al., 2005; Dall'Osto et al., 2006). A large percentage of the carotenoids *in situ* (from 80 to 95%, according to some studies) are bound to protein complexes and their dissociation from the proteins occurs very rarely, as in the case of the de-epoxidation of violaxanthin to zeaxanthin during the xanthophyll cycle (Tardy and Havaux, 1997). Lutein is a key structural component in the largest subunits of the light-harvesting complexes and, more specifically Lhcb1-3, which is a part of the trimer complex of LHCII (Liu et al., 2004; Standfuss et al., 2005). Each one of the subunits of LHCII is composed of a polypeptide chain, containing three trans-membrane α-spirals and multiple chlorophyll molecules (8 molecules of chlorophyll *a* and 6 molecules of chlorophyll *b*) are associated to each subunit (Fig. 5). In addition, four xanthophyll molecules are part of the structure of these subunits and each of them binds to a strictly specific place. Two of these four xanthophylls are lutein molecules. In addition, lutein is essential for the correct three-dimensional folding of the antennae proteins (Plumley and Schmidt, 1987; Paulsen et al., 1990). If not sufficient amount of lutein is available, the subunits can bind with other xanthophylls such as zeaxanthin and violaxanthin, but the formed complex is too unstable and its trimerisation is inhibited (Jahn et al., 2001; Dall'Osto et al., 2006; Werner et al., 2006). These facts show the important significance of lutein in the structure of the photosynthetic apparatus. The trimeric subunits of the light-harvesting complex contain one molecule neoxanthin and a molecule from the xanthophyll cycle (zeaxanthin or violaxanthin). The content of neoxanthin is relatively constant in higher plants (about 9-14% of total carotenoid content) (Johnson et al., 1993; Bungard et al., 1997). Other antenna proteins also include xanthophyll molecules in their composition, but in different amounts. For example, large proteins from LHCII and LHCI bind 14 chlorophyll molecules, however chlorophylls are comparatively less in the monomeric proteins from

LHCII, Lhcb4-6 (CP24, CP26, CP29). Furthermore, the proteins from LHCII can bind up to 4 xanthophylls, whereas the other antennae proteins can bind two or three (Standfuss et al., 2005; Amunts et al., 2007).

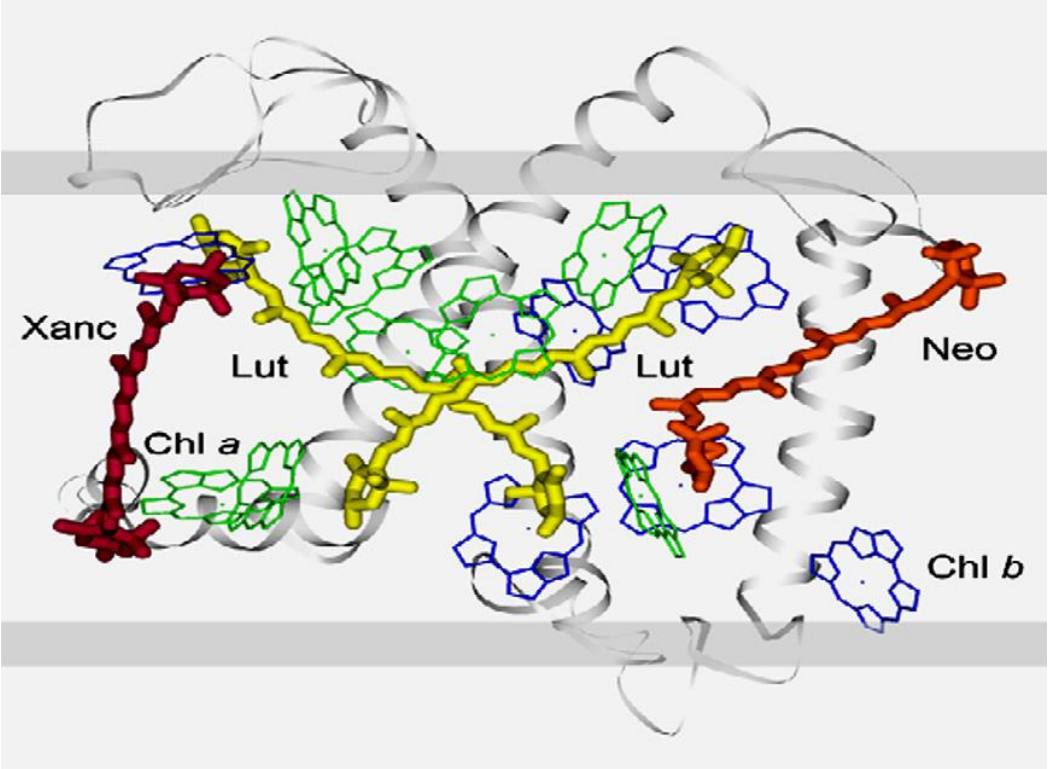

Fig. 5 Crystallographic structure of one of the trimeric subunits of LHCII. Chl. a has a green colour, chl. b is blue. Both lutein molecules are coloured in yellow, neoxanthin is orange, and the violoxanthin/zeaxanthin is dark red. (Jahn et al., 2012; Liu et al., 2004)

Perhaps one of the most important functions of lutein, concerning the photoprotection is quenching of the triplet chlorophyll forms (Dall'Osto et al., 2006; Mozzo et al., 2008). These authors have confirmed, that the neoxanthin and violaxanthin are much less effective in quenching the triplet forms of chlorophyll and that the most effective is the quenching by lutein, when it is associated with the antennae proteins. The substitution of the lutein inside antennae complexes with other type of xanthophyll leads to highly increased photoinhibition. Other function of lutein is to capture the light energy and transport it to the chlorophyll molecule (Siefermann-Harms, 1985). Nevertheless, the violaxanthin is more effective light-harvesting pigment than lutein and can entirely substitute it in this function (Pogson et al., 1996; Kalituho et al., 2007). The lutein-epoxide, which is a part of the so-called lutein cycle captures light energy also more efficiently than lutein (Matsubara et al., 2007; Garcia-Plazaola et al., 2007; Matsubara et al., 2009). It appears, that some xanthophylls are self-replaceable,

but this substitution is limited to specific functions only (lutein-violaxanthin) or to a certain degree (as in the case with lutein and zeaxanthin in structural relation) (Jahn et al., 2012). The function of xanthophylls is determined not only by their chemical composition as well as structure, but also by their binding in complexes with proteins (the antenna proteins). It is widely accepted, that lutein acts as a quencher of the excited singlet forms of the chlorophyll, but this function is not related to the non-photochemical quenching (Pogson et al., 1998; Lokstein et al., 2002). According to some new literature data, the role of lutein in this type of quenching is insignificant, because the excessive unused energy is re-distributed amongst the chlorophyll molecules (Muller et al., 2010).

Zeaxanthin is one of the three pigments from the xanthophyll cycle (along with the violaxanthin and antheraxanthin). This cycle acts in the process of quenching of ROS and protects from photodamage after abiotic stress and intense light (Sapozhnikov et a, 1957; Yamamoto et al., 1962). The zeaxanthin itself is extremely important for the normal function of thylakoids, as well as the reactions from the light phase of photosynthesis, especially after photodamage by certain types of abiotic stresses (Havaux and Niyogi, 1999). Due to its structure, zeaxanthin increases the membrane fluidity, attaching to both sides of the membrane with the two polar ends, whereas the non-polar part remains in the centre of the membrane (Tardy and Havaux, 1997). Thus, it increases membranes stability and strengthens the impermeability of the membranes to oxygen, protecting their sensitive hydrophobic parts from the oxidative action of ROS (Gruszecki and Strzałka, 2005). These properties make its physiological action very similar to the cholesterol inside membranes of animal cells. The latest literature data (Demmig-Adams et al., 2012) recently showed, that carotenoids together with tocopherol also affect the synthesis of oxylipins and oxylipin hormones (jasmonates), deactivating the ROS, which have been accumulated as a result from the negative effect of light. The greatest photoprotective properties of all carotenoid pigments, are attributed to the zeaxanthin (Havaux et al., 2007). This is mainly due to its chemical structure that includes two polar rings with function, described briefly earlier. Zeaxanthin molecule has a non-polar body with a system of 11 conjugated double bonds, explaining its light absorption properties. In contrast, lutein and antheraxanthin have both 10 such double bonds, violaxanthin has nine and neoxanthin has seven. It is considered, that the higher the amount of these double bonds, the greater the photoprotective properties of the molecule (Woodall et al., 1997; Gruszecki, 1999, 2004; Gruszecki and Strzalka, 2005). These properties are amplified after binding in complex with the subunits of LHCII. There, it can quench directly the triplet chlorophyll and singlet oxygen species, formed in P680. It was found out, however, that zeaxanthin can take part in photoprotection enough actively even without binding to the LHCII (Havaux et al., 2007).

According to the commonly accepted opinion, the increased zeaxanthin content after light stress (combination of temperature stress and photodamage), is due to the reversive conversion of the violaxanthin into zeaxanthin (de-epoxidation of violaxanthin) as a component from the xanthophyll cycle. In normal conditions and in absence of stress, zeaxanthin turns into violaxanthin by the means of the enzyme zeaxanthin epoxidase (ZEP or ABA1, mutant forms of this enzyme in *A. thaliana*). This reaction occurs in the stroma of chloroplasts at pH 7 and in the presence of O_2. In stress conditions, violaxanthin is converted to zeaxanthin by the reverse reaction called de-epoxidation, which takes place in the lumen of the thylakoid membranes, at pH 5.2 by the enzyme violaxanthin de-epoxidase. During this reaction the violaxanthin is dissociated from the antenna complex, and after the formation of zeaxanthin it is possible to be inserted in the light-harvesting complexes again, in the appropriate conditions (Tardy and Havaux, 1997). It is well-known, that zeaxanthin has a greater capacity than violaxanthin for neutralization of the ROS, which accumulate in high amounts after freezing stress, especially in combination with photodamage. The presence of free carotenoids inside the membrane leads to increase in its stability and limits the lipid peroxidation (Lim et al., 1992; Sarry et al., 1994; Havaux et al., 1996). Furthermore, the singlet electron forms of zeaxanthin has lower energy, even than chlorophyll, which allows the unused light energy to be quenched non-photochemically (dissipating it in a form of heat) (Frank et al., 1994). This is not true for violaxanthin, whose energetic forms are higher than chlorophyll and thus it can act as a light-harvesting pigment, but not as a quencher of energy. In some plants, a conversion cycle of carotenoids exist, similar to the xanthophyll cycle, when the light is in excess. It is called the lutein cycle and it includes the participation of lutein, which consequently epoxygenates at low intensity of light, and de-epoxygenates at high intensity of light, similarly to the conversion of zeaxanthin to violaxanthin and vice versa. This demonstrates the role of lutein as a pigment, quenching the unused energy in a similar way like the zeaxanthin (Bungard et al., 1998).

Tocopherols

Tocopherols are terpenoid compounds that have the function of antioxidants. They are widely distributed in nature and take place in the antioxidant defence system of organisms. α-tocopherol is one of the most well-known of them under the name vitamin E. Tocopherols participate in the neutralization of the reactive oxygen species, mostly the singlet oxygen formed in PS II of thylakoids. Tocopherols are a component from the thylakoids of chloroplasts as well, because they have a lipid nature and their hydrophobic (prenyl) groups can attach to the membranes. They are synthesised from the shikimate pathway in plants and exist in four forms, depending on the number and location of the

methyl groups, attached to their hydroquinone ring (α, β, γ, δ). They are involved in the regulation of the formation of jasmonates from the peroxidation of lipids in the thylakoid membranes in plants after stress.

The main function of tocopherols is their antioxidant action. It is realized mostly in quenching and deactivation of the singlet oxygen forms, the lipid peroxyl radicals, neutralizing the heavy metal ions etc. (Szarka et al., 2012). α-tocopherol molecule has the ability to quench (only in physical way by resonance energy transfer) averagely about 120 molecules of singlet oxygen before undergoing oxidation by chemical deactivation (Fahrenholz et al., 1974; Munne-Bosch and Alegre, 2002). The reactive oxygen species, that form after stress attack mainly the membrane system of plant cell, mostly the sensitive thylakoid membranes in chloroplasts. Initially, lipid peroxyl radicals form in this process. α-tocopherol neutralizes these radicals converting itself into tocopheroxyl radical, and lipid peroxydes into lipid hydroperoxides (Kamal-Eldin and Appelqvist, 1996). After that tocopheroxyl radical can be regenerated from the antioxidant system of the cell, a conjugation of tocopherol, ascorbate and glutathione molecules, in the process of their mutual regeneration (Szarka et al., 2012). The ascorbate and glutathione themselves are very strong antioxidants and have the ability to reduce the hydrogen peroxide, that forms from ROS after stress. In the process their oxidized forms are produced re-generating (reducing) themselves by the means of so-called antioxidant system, the tocopherol being reduced by ascorbate, ascorbate by the glutathione and glutathione by NADPH. However, in case the amount of some of these compounds is insufficient, tocopherol cannot regenerate and oxidized tocopherol products form, that cannot be regenerated to tocopherol (Kamal-Eldin and Apelqvist, 1996). During chemical deactivation of the singlet oxygen tocopherol radicals form as well (tocopherol quinones etc.), that cannot be subjected to regeneration from the antioxidant system of plant cell (Munne-Bosch and Alegre, 2002). Normally, in tolerant to stress plants the content of tocopherol increases significantly, whereas in sensitive plants it decreases after stress (Munne-Bosch, 2005). Furthermore, in tolerant plants the amount of tocopherols decreases after intensive and prolonged stress exposure (Boo and Jung, 1999). Many transgene plants which express high levels of the genes for biosynthesis of tocopherols have increased tolerance to stress, for example increased tolerance to drought in tobacco (Liu et al., 2008), to cold stress in Arabidopsis (Maeda et al., 2006), tolerance to osmotic stress in tobacco (Abbasi et al., 2007). In the last case it was found, that plants with increased expression of α- or γ-tocopherol have different resistance to different types of stress. In the same way in plants, showing a deficit in α-tocopherol a lower tolerance to stress was observed, for example to heavy

metals in Arabidopsis (Collin et al., 2008). The very content of tocopherol in the cell is a result of balance between synthesis, regeneration and degradation. Therefore, regardless of the high antioxidant and protective function of tocopherols, their defensive function is limited by the presence of other antioxidants in the cell and by other defence mechanisms, as well as by the duration and intensity of the stress factor (Szarka et al., 2012).