N-glycosylation regulates photosynthetic efficiency of Arabidopsis thaliana


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Abstract

N-glycosylation is one of the most important protein modifications in eukaryotes. It has been well established that N-glycosylation plays multiple roles in regulating stress tolerance of plants. However, the effects and mechanism of N-glycosylation on photosynthesis have not been well understood. In the present study, an obvious decrease in photosynthetic capacity and dry mass were detected in alg3-3 and cgl1-1, two typical mutants in N-glycosylation process. The maximal photochemical efficiency of PSII decreased significantly in cgl1-1. The values of effective quantum yield of PSII photochemistry, rate of photosynthetic electron transport through PSII, and photochemical quenching coefficient, which reflected the photochemical efficiency of plants, decreased as well, while the values of quantum yield of nonregulated energy dissipation of PSII showed obvious enhancement, the similar tendency was also observed in alg3-3. Furthermore, we found that N-glycosylation was also required to maintain the stability of a chloroplast-located protein CAH1, which was closely related to photosynthesis. These results suggest that N-glycosylation plays crucial roles in maintaining photosynthetic efficiency.

Additional key words: asparagine-linked glycosylation; biomass; carbonic anhydrase; chlorophyll fluorescence.

Introduction

Asparagine-linked glycosylation (N-glycosylation) is an essential process occurring in the lumen of the endoplasmic reticulum (ER) and the secretory system of eukaryotic organisms (Silberstein and Gilmore 1996, Helenius and Aebl 2001). It is critical for protein folding, quality control, and transport of the secretory proteins, which plays crucial roles in plant adaptive responses to environmental stresses, such as plant immunity (Kang et al. 2015), temperature tolerance (Zhang et al. 2009), and salt sensitivity (Koiwa et al. 2003, Liu et al. 2018). More than one thousand proteins containing different N-glycosylation sites have been found in the model plant Arabidopsis thaliana (Song et al. 2013). As one of the major and most widespread protein posttranslational modifications in eukaryotes, the core processes are evolutionarily strongly conserved.

The initial synthesis of the lipid-linked oligosaccharide occurs on the cytoplasmic side of the ER and is mainly carried out by a series of glycosyltransferases of the ALG (asparagine-linked glycosylation) family (Bickel et al. 2005). The precursor is then transported across the ER membrane by a flippase-like protein for further synthesis to finally form an oligosaccharide containing three molecules of glucose (Glc), two molecules of N-acetylglucosamine (GlcNAc), and nine molecules of mannose (Man) in the luminal side of the ER (Higgins 1994, Stagljar et al. 1994, Reiss et al. 1996, Larkin and Imperiali 2011, Schoberer et al. 2018). Subsequently, the completed oligosaccharide (Glc1Man1GlcNAc2) is transferred by the oligosaccharyltransferase (OST) complex onto the asparagine in the Asn-Xaa-Ser/Thr consensus motif (Xaa represents any amino acid except proline) of the nascent polypeptides (Silberstein and Gilmore 1996, Helenius and Aebl 2001). The mannose glycans are gradually trimmed as part of ERQC (ER-associated quality control compartment) system (Aebi et al. 2010). Then, the correctly folded glycoproteins are transported to the Golgi apparatus and further modified by Golgi resident glycosyltransferases and glycosidases for the formation of glycoproteins with mature oligosaccharide (Song et al. 2013, Strasser 2016).

The significance of N-glycosylation modifications in mammals has been well exemplified by the severe congenital disorders resulting from mutations of the N-glycosylation pathway (Schollen et al. 2005). However, no visible change was found in the Arabidopsis N-glycosylation mutant alg3, which is defective in the biosynthesis of the dol-PP-linked glycans; it seems reasonable to suggest that the glycans in the structure of mannose type were shown to be nonessential (Henquet et al. 2013, Strasser 2016). The degradation of the luminal N-glycosylation pathway in the ER could lead to a reduction in an asparagine-linked glycosyltransferase (Aebi 2001). It is critical for protein folding, quality control, and transport of the secretory proteins, which plays crucial roles in plant adaptive responses to environmental stresses, such as plant immunity (Kang et al. 2015), temperature tolerance (Zhang et al. 2009), and salt sensitivity (Koiwa et al. 2003, Liu et al. 2018). More than one thousand proteins containing different N-glycosylation sites have been found in the model plant Arabidopsis thaliana (Song et al. 2013). As one of the major and most widespread protein posttranslational modifications in eukaryotes, the core processes are evolutionarily strongly conserved.

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N-Glycosylation regulates photosynthesis in Arabidopsis thaliana, the complex glycan less mutant (cgl1-1), which completely lacks the complex N-glycans, has also no visible developmental disorders and only shows some changes in salt-stress response (Kang et al. 2011). The phenotype ambiguity of N-glycosylation mutants in 'ideal' greenhouses may be the reason why this process is rarely studied in plants (Zielinska et al. 2012). Nevertheless, the N-glycosylation abnormalities result in unfolded protein response (von Schaewen et al. 1993, Henquet et al. 2008). This reminds us that many unknown abnormalities are still to be discovered. It is well known that photosynthesis in plants and algae occurs in chloroplasts, and this process is the basis of plant growth and development. In addition, most chloroplast-located proteins are synthesized in the ER and then transported to the chloroplast through the secretory pathway. CAH1, alpha-type carbonic anhydrase (EC 4.2.1.1), is one of the few plant proteins known to be targeted to the chloroplast, and which is essential for efficient photosynthesis (Samuelsson and Karlsson 2001, Burén et al. 2011, Kupriyanova et al. 2017). The mature CAH1 harboring multiple glycoforms of N-glycans (Burén et al. 2011) might be a good substrate to study the relation between N-glycosylation and photosynthesis.

It has long been known that chlorophyll (Chl) fluorescence induction kinetics of leaves from plants provides an indicator of plant photosynthetic capacity. The fluorescence parameters are directly related to the energy transport, the photosynthetic carbon dioxide assimilation rate, and the formation of primary photosynthetic sugars in plants (Harbinson et al. 1990, Murchie and Lawson 2013). As rapid and noninvasive technique for detecting subtle differences of leaf metabolism or developmental disorders of the seedlings (Barbagallo et al. 2003), Chl fluorescence induction kinetics has received increasing attention in the fields of plant physiology, molecular biology, and molecular genetics.

In the present study, we analyzed the phenotype of ALG3 and CGL knockout mutant plants. We showed that the mutants grow normally under greenhouse conditions. In order to investigate whether N-glycosylation could affect the photosynthetic capacity, we also examined if chlorophyll fluorescence parameters could reveal the possible alterations in the PSII photochemistry in the alg3-3 and cgl1-1 mutants. Our data showed that the photosynthetic capacity of alg3-3 and cgl1-1 decreased to different degrees. Moreover, the underlying molecular mechanisms of photosynthetic capacity changes were also analyzed.

Materials and methods

Plant materials and growth conditions: Arabidopsis thaliana, ecotype Columbia (Col-0), was the parental line for mutant plants and the generation of transgenic plants. Seeds of alg3-3 (SALK 046061) and cgl1-1 (CS6192) were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. The seeds were surface sterilized with 70% ethanol and then stratified at 4°C for 2 d in darkness. The stratified seeds were placed on plates containing 1/2 MS medium supplemented with 0.3% gelrite and 1% sucrose. The plates were then placed for seed germination and plant growth in a growth chamber (MLR-352H-PC, Panasonic, Japan) at 22°C under irradiance of 100 ± 5 μmol(photon) m⁻² s⁻¹ at long-day conditions with a 16-h light/8-h dark cycle, and 50% relative humidity.

Chl fluorescence parameters were measured using a portable fluorometer (Mini PAM, Walz, Germany), as described previously by Demmig-Adams et al. (1996). The PSII maximal photochemical efficiency (Fv/Fm) was defined as (Fm − F0)/Fm, where Fm is the maximum fluorescence emission from the dark-adapted state measured with a pulse of saturating light, and F0 is the minimal fluorescence from the dark-adapted state. The effective quantum yield of PSII photochemistry (ΦPSII) was defined as (Fm − Fv)/Fm, where Fm is the maximum fluorescence emission from the light-adapted state measured with a pulse of saturating light and Fv is the minimal fluorescence of the light-adapted state measured with a far-red pulse. The NPQ parameter was defined according to the equation: NPQ = (Fm – Fv)/(Fm – Fv'), where Fv' is minimal fluorescence of the light-adapted state measured with a far-red pulse. The coefficient of photochemical quenching (qP) was defined as (Fm' – Fv')/(Fv' – Fv') according to Lazár (2015). As ΦPSII represents the number of electrons transferred per photons absorbed by PSII, the ETR (the rate of photosynthetic electron transport through PSII) can be calculated as ETR = ΦPSII × PAR × 0.5 × 0.84 according to Genty et al. (1989). The quantum yield of nonregulated energy dissipation of PSII (Y(NO)) and the quantum yield of regulated energy dissipation of PSII (Y(NPQ)) was also defined according to Kramer et al. (2004).

Dry biomass and leaf Chl content: For dry biomass measuring, shoots of 4-week-old plants were detached and oven-dried at 80°C for 24 h; the shoots were then weighed separately. The leaf Chl content was measured spectrophotometrically as described previously with minor modifications (Liang et al. 2017). Fresh leaves were weighed and incubated at room temperature with 10 mL of solution (acetone: ethanol = 1:1) for at least 24 h, then clarified by centrifugation for 5 min at 13,000 × g, while this process was protected from light. The absorbance of the supernatant was measured at wavelengths 645, 652, and 663 nm (A₆₄₅, A₆₅₂, and A₆₆₃) with the microplate reader (FL-600, BioTek, USA). Chl concentration was estimated using following equations: Chl a [mg g⁻¹] = (12.7 × A₆₄₅ – 2.69 × A₆₅₂) × V/(1,000 × W), Chl b [mg g⁻¹] = (27.9 × A₆₆₃ – 4.68 × A₆₄₅) × V/(1,000 × W), total Chl [mg g⁻¹] = (A₆₄₅ + A₆₅₂ + A₆₆₃)/(34.5 × W), where V represents the volume [mL] of the extracted solution, and W represents the fresh mass [g] of the sample.

Identification of the homozygous mutant plants: Genomic DNA was extracted from the leaves as the template for PCR reaction. To identify the genotype of the T-DNA insertion alg3-3 mutant, the DNA was amplified with the forward primer alg3-F1 GATGCAATCCTAGTCGCACT;
reverse primer alg3-R1 CCTGAGCAGGTTAGACTTCC, and the T- DNA left border primer L Ba1 TGGTTACACG-TAGTGGCCCATCG. To identify the genotype of the alg3-1 mutant, the primer alg1-1-dcaps-F2 TATCAAACCT-CAGGCAGTTCT, and primer alg1-1-dcaps-R2 GACAGCTTGTACAGATCATG were used for PCR amplification. The PCR products were digested with Hinf I (TaKaRa Bio, Japan) and then electrophoresed.

Transgenic plant construct: pCAH1: GFP was generated from pfPZ221-BRI1: BRI1-GFP as follows. The primers 5'-ATGGTACG-TGAGCAGGGTAGACTTCC (0.05% CBB, 10.0% acetic acid, and 5.0% B): was generated a glycan in Golgi body (Fig. 1), compared with that of WT. These changes could mutant plants compared with the WT plants, PSII (Fig. 2). Nevertheless, a dry biomass accumulation decreased significantly in the mutant plants compared with the wild type (WT) (Fig. 1S, supplement).

N-glycosylation defects induce changes in Fv/Fm: The Chl fluorescence parameter, Fv/Fm, reflects the maximum quantum efficiency of PSII photochemistry in the dark-adapted state and it has been widely used for early stress detection in plants (Schansker et al. 2014, Magyar et al. 2018). Our results showed that the value of Fv/Fm declined differently in the typical N-glycosylation-deficient alg3-3 and alg1-1 mutant plants compared with the WT plants, especially in the alg1-1 mutant, whose values were significantly lower than those in the leaves of WT (Fig. 2). Thus, the above data suggested that their photosynthetic capacity was damaged to a certain degree.

Biochemical analyses: Arabidopsis seeds were germinated on 1/2 MS medium. To inhibit N-glycosylation, 2-week-old seedlings were transferred to 1/2 MS medium containing 30 μg mL−1 tunicamycin (Sigma) for continued growth for another 24 h in the growth chamber as described previously with minor modifications (McCormack et al. 2015). The samples were prepared for loading by adding 2 × sample buffer [0.125 M Tris (pH 6.8), 4.0% SDS, 20% glycerol, 0.2 M DTT, 0.02% bromophenol blue], and heating the samples at 95°C for 10 min. Then the supernatants were used for SDS-PAGE on 8.0% Tris-acetate (Liu et al. 2018). Next, proteins in the gel were electrothermally transferred to PVDF membrane (Roche, Switzerland). Membranes were incubated with primary antibodies in 5.0% milk at room temperature. The primary antibody used in this study was mouse anti-GFP (Abcam); the secondary antibody was HRP-conjugated goat anti-mouse IgG (D110087, BBI). Rubisco small subunit (RbcS) was used as a loading control. The SDS-PAGE containing RbcS was stained with Coomassie Brilliant Blue R-250 (0.05% CBB, 10.0% acetic acid, and 5.0% methanol) for 2 h and then destained by destaining solution (7.0% acetic acid and 5.0% methanol). The relative amount of proteins was determined by stained RbcS bands separated by SDS-PAGE (Dyballa and Metzger 2009).

Statistical analysis: The results were expressed as the mean ± standard deviation. The data were statistically evaluated with one-way analysis of variance (ANOVA) between the different groups and by Tukey’s multiple comparisons test. The difference was considered to be statistically significant when P<0.05.

Results
alg3-3 and alg1-1 showed inconspicuous phenotype under optimal conditions in greenhouse: alg3-3 is a T-DNA insertion mutant identified in Arabidopsis (Fig. 1A), it derives from incidental splicing of the third exon carrying the T-DNAs, which blocks the first mannosae addition step on Dol-PP-linked glycans in ER (Fig. 1C). Besides, we also investigated another Arabidopsis alg3-1 mutant that disrupts adding a GlcNAc residue to the ManGlcNAc: glycan in Golgi body (Fig. 1B, C). Both of these homozygous plants did not show any visible changes in their phenotype under normal laboratory conditions (Fig. 1C). Nevertheless, a dry biomass accumulation decreased significantly in the mutant plants compared with the wild type (WT) (Fig. 1S, supplement).

Photochemical efficiency decreased in alg3-3 and alg1-1: In order to investigate further the effects of N-glycosylation on actual photochemical efficiency of plants, correlation parameters for photochemical efficiency were measured and analyzed. The Φpsii denotes the effective quantum yield of PSII photochemistry for the light-adapted state, also denoted as effective quantum yield of PSII photochemistry, which is an indicator of the real photochemical activity (Genty et al. 1989). In the present study, the data clearly showed that the values of Φpsii decreased obviously with the increase of light intensity both in the mutant plants and the WT (Fig. 3A). However, it is worth mentioning that the mutants always exhibited lower Φpsii values than that of the WT under the same irradiance (Fig. 3A). The qP (Fig. 3C), which estimates a fraction of ‘open’ PSII reaction centers based on a puddle model (Stribet 2013, Lazár 2015), had the same tendency of change as that of the Φpsii. Similarly, the photochemical efficiency and the rate of photosynthetic electron transport through PSII (ETR) also showed a downward trend in the mutants (Fig. 3B), compared with that of WT. These changes could give a good indication that the defective of N-glycosylation led to a decrease in the photosynthetic capacity of plants.

Nonphotochemical quenching in alg3-3 and alg1-1: Nonphotochemical quenching parameter (NPQ) equals the ratio of the quantum yield of regulated energy dissipation to the quantum yield of nonregulated energy dissipation of PSII (Lazár 2015). In order to further investigate whether nonphotochemical processes were affected in the mutant plants, the coefficient of nonphotochemical quenching (qN), the NPQ parameter (Fig. 4A, B), and the quantum yield of regulated energy dissipation in PSII, Y(NPQ) (Fig. 4C), were analyzed. The nonphotochemical processes rose along with the increase of light intensity, slight changes still could be found in qN and Y(NPQ), while the NPQ values were always lower than their counterparts in WT,
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especially, in the cgl1-1 mutant (Fig. 4B). These results indicated that N-glycosylation also affected nonphotochemical processes.

Y_{(NO)} reflects the yield for other energy losses (Lazár 2015); in most cases, high Y_{(NO)} value indicates that both photochemical energy conversion and protective regulatory mechanisms are inefficient. In other words, plants have serious problems to cope with the incident radiation, either it had been already damaged or it would be photodamaged upon further irradiation (Kramer et al. 2004). Thus, contrary to previous results, the values of Y_{(NO)} showed a clear increase in the mutants, especially in cgl1-1 (Fig. 4D). These results indicated that the N-glycosylation defect mutants suffered more of PSII damage than WT.

Carbonic anhydrase degradation after deglycosylation:
It has been well established that carbonic anhydrase (CAH1) influences the activity of carboxylase and decarboxylase, which is essential for efficient photosynthesis capacity of the plant (Badger and Price 1994). The accurate localization and stable existence of protein is the premise of its normal physiological function. Our results showed that the stabilization of CAH1 was influenced by the treatment of tunicamycin (an antibiotic inhibiting N-glycosylation) (Fig. 5A). Our finding suggested that degradation of CAH1 might be the reason, which led to a decrease of photosynthesis in the N-glycosylation defect mutant plants (Fig. 5B).
A and \( \). These data indicated that capacity \( \text{ETR} \) with the increase of light intensity. Each curve \( \text{ETR} \) did not show any visible \( \text{ETR} \). However, many studies have shown that the \( \text{ETR} \) did not show any visible \( \text{ETR} \). These values decreased much \( \text{ETR} \) and \( \text{ETR} \). The results intensely demonstrated \( \text{ETR} \) \( \text{ETR} \). As one of the most common and essential protein modifications, N-glycosylation plays multiple roles in eukaryotes. Previous research on this process focused on mammals or yeast, because the mutant plants exhibited rarely any visible phenotype changes under normal conditions. Recently, thousands of different N-glycosylated proteins have been identified in Arabidopsis thaliana (Zielinska et al. 2012, Song et al. 2013), indicating that glycosylation defects have also wide-ranging effects in plants. In recent years, many studies have shown that Chl fluorescence could represent imperceptible changes in the operation and function of the photosynthetic apparatus, even the stress tolerance of plants.

The results of the present study showed a substantial reduction of the photosynthetic capacity in the N-glycosylation mutant plants. However, the PSII activity, reflected by the \( F_\text{m}/F_\text{m} \), seemed to be affected slightly in \( \text{alg}3-3 \) (Fig. 2). This result may suggest that \textit{in vitro} measurements of PSII activity using isolated chloroplasts or thylakoid membranes might not be necessarily equivalent to PSII photochemical activity \textit{in vivo} (Lu and Zhang 1998, Schansker et al. 2014). Homozygous \( \text{alg}3-3 \) and \( \text{cgl}1-1 \) plants (Fig. 1A,B) did not show any visible phenotype alterations under normal laboratory conditions (Fig. 1C). However, many studies have shown that the glycosylation abnormalities result in some disturbance in the cellular level, such as the provoking of the unfolded protein responses in the ER (von Schaewen et al. 1993, Henquet et al. 2008). This was in agreement with our observation of the significant decrease of dry biomass in both \( \text{alg}3-3 \) and \( \text{cgl}1-1 \) (Fig. 1S).

The effective photochemical quantum yield of PSII (\( \Phi_{\text{psII}} \)) and \( q_p \) gradually decreased with the increase of light intensity (Fig. 3A). These values increased much faster in the mutants compared to that in WT, while the \( \text{ETR} \) and the NPQ presented a remarkable rise with increasing light intensity and the values of parameters in the mutants were lower than that of the WT at the same light intensity (Fig. 4A,B). These data indicated that capacity of capture (and transfer) of the excitation energy to the photosynthetic reaction centers decreased with increasing light intensity (Green and Parson 2003). However, with the increase of light intensity, the excess excitation energy is dissipated through nonphotochemical quenching (NPQ) of the excited state of Chl \( a \) in the form of heat (Ort 2001, Demmig-Adams et al. 2014). Values of NPQ decreased in both \( \text{alg}3-3 \) and \( \text{cgl}1-1 \) mutants (especially in \( \text{cgl}1-1 \)) compared to the WT (Fig. 4B), indicating the mutant plants may experience damage to PSII (Kozi 2006), either on the (electron) donor or the (electron) acceptor side of PSII (Pospíšil 2009, Hamdani et al. 2019).

Although plants have developed a number of photo-protection responses such as nonradiative dissipation of energy as heat at the molecular level (Müller et al. 2001, Derks et al. 2015), too much light can affect PSII and leads to damage and degradation of the reaction center (Barber and Andersson 1992). \( Y_{\text{NO}} \) represents basal (= nonregulated) nonphotochemical quenching, opposite to \( Y_{\text{NPQ}} \), which represents regulated, light-induced nonphotochemical quenching (Lázár 2015). Along with the decreased photosynthetic capacity in the mutant plants, significant growth in \( Y_{\text{NO}} \) was observed in \( \text{alg}3-3 \) and \( \text{cgl}1-1 \) (Fig. 4D). The results intensely demonstrated that such a decrease in photochemical efficiency was at least partly associated with the significant increase of the nonphotochemical quenching, which was not photoprotective, or with photosystem damage caused by high-light stress.

Chloroplasts are organelles of endosymbiotic origin and the vast majority of chloroplast proteins are synthesized in a precursor form on cytosolic ribosomes, while a unique set of protein transport machinery is responsible for the import of the nuclear-encoded proteins into the chloroplast (Jarvis and Soll 2001, Gutensohn et al. 2006). Secretory proteins are mostly modified by N-glycans and

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**Discussion**

As one of the most common and essential protein modification processes, N-glycosylation plays multiple roles in eukaryotes. Previous research on this process focused on mammals or yeast, because the mutant plants exhibited rarely any visible phenotype changes under normal conditions. Recently, thousands of different N-glycosylated proteins have been identified in Arabidopsis thaliana (Zielinska et al. 2012, Song et al. 2013), indicating that glycosylation defects have also wide-ranging effects in plants. In recent years, many studies have shown that Chl fluorescence could represent imperceptible changes in the operation and function of the photosynthetic apparatus, even the stress tolerance of plants.

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The processing of N-glycans in the ER determines the fate of associated secretory peptides (Helenius and Aebi 2001). It is well documented that abnormal N-glycans in ER are detected by ERQC (ER-associated quality control compartment) system and misfolded proteins are removed via the ERAD (ER-associated degradation) pathway (Hong *et al.* 2009, Aebi 2013, Strasser 2016). Previous reports suggest that a chloroplast-located protein CAH1 becomes N-glycosylated before entering the chloroplast through the secretory pathway (Villarejo *et al.* 2005, Burén *et al.* 2011), indicating that N-glycosylation plays the role in regulation of secretory pathway for chloroplast-located proteins. The well N-glycosylated CAH1 is transported into the chloroplast through the secretory pathway in the WT plants and then participates in the process of photosynthesis. On the other hand, the misfolded or damaged CAH1 (caused by perturbation of N-glycosylation) in the mutant plants might activate the ERQC/ERAD machinery, which may finally lead to the degradation of CAH1 before entering the chloroplast (Fig. 5B). In addition, CAH1 degraded obviously after deglycosylation by tunicamycin (Fig. 5A). These findings suggested that the decline of photosynthetic capacity in the *alg3-3* or *cgl1-1* mutant may be also a consequence of the functional decomposition of CAH1 caused by N-glycosylation defects. Moreover, it is worth mentioning that the calculated significant decrease in dry biomass of *alg3-3* and *cgl1-1* (Fig. 1S) was thus consistent with the findings of the reduced photosynthetic rate, although the Chl content barely changed (Fig. 2S, supplement).

N-glycosylation is essential for multicellular life and its complete absence is embryonically lethal. In this work, we found that the obviously decreased photosynthetic capacity in N-glycosylation defective mutants *alg3-3* and *cgl1-1* might be associated with the disturbance of the PSII and impaired electron transfer system. Based on the above results, we also showed that N-glycosylation...
regulated the photosynthetic capacity of plants at least in part by CAH1, although the exact mechanism(s) must be confirmed. In conclusion, N-glycosylation is required for plants to maintain the normal function of photosynthesis.

References


