Functional aspects of baculovirus DNA photolyases

Fang Xu

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Abstract

Baculoviruses are insect viruses that are applied as biological control agents due to adequate virulence, host specificity and safety for the environment. Solar light negatively affects field performance of baculoviruses by reducing their infectivity, most likely as a consequence of the formation of cyclobutane pyrimidine dimers (CPDs) in the viral DNA upon ultraviolet (UV) irradiation. CPDs can be repaired by CPD photolyases when exposed to blue light photons, a process called photoreactivation. From previous work it was known that the *Cc-phr2* gene of the baculovirus *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) encodes a biochemically active photolyase. The research in this thesis focuses on (i) the degree of conservation of CPD photolyase (*phr*) genes in a subgroup of baculoviruses, (ii) the localization of baculovirus photolyase proteins in insect cells and occlusion derived virus (ODV), and (iii) the *in vivo* effect of *phr* genes on the UV sensitivity of baculoviruses. Homologues of the Cc-*phr* genes were found in all studied group II NPVs in the genus Alphabaculovirus that infect insects in the subfamily Plusiinae insects. Phylogenetic analysis suggested that these *phr*-like genes have a common ancestor. Intracellular localization of the two ChchNPV encoded PHR proteins in insect cells was studied using enhanced GFP fusion. Both PHR1 and PHR2 localized in the nucleus and associated with chromosomes, spindle, aster and midbody structures during host cell mitosis. Moreover, Cc-PHR2 co-localized with virogenic stroma, when PHR2-EGFP-transfected cells were infected with *Autographa californica* (Ac) MNPV. Neither of the two Cc-PHR proteins was identified by LC/MS-MS in the ODVs of ChchNPV. To evaluate the potential of the Cc-PHR2 protein to reduce the UV sensitivity of a baculovirus, the *Cc-phr2* gene was incorporated in the genome of *Helicoverpa armigera* (Hear) NPV, which does not have a UV damage repair system. This resulted in a decreased sensitivity to UV-light compared to wild type HearNPV. A cell line was established from embryos of the insect *C. chalcites*. This cell line was shown to be permissive for both ChchNPV and the related *Trichoplusia ni* NPV (TnSNPV). This novel cell line will be a useful tool for making ChchNPV *phr* mutant viruses to study the impact of DNA repair mediated by photolyases on baculovirus ecology. The collected data support the hypothesis that the Cc- *phr2* gene provides a baculovirus with an ecological benefit by increasing the resistance to UV.

Contents

Chapter 1

General introduction

Introduction

Baculoviruses form a large family of DNA viruses which specially infect arthropods, mainly lepidopteran insects. These viruses are used broadly as (i) biological control agents against insect pests, (ii) as expression vectors of foreign proteins and (iii) as gene delivery vectors for mammalian cells (gene therapy). In agronomy baculoviruses are used to control certain insect pests as alternatives to chemical insecticides. The use of baculoviruses is now taking place for over half a century without negative effects on the environment or the producer / consumer. A major downside of using baculoviruses in the field is their sensitivity to UV irradiation. As a consequence UV protectants are widely used as part of the spray formulation. Recently, a potential UV repair system has been identified in baculoviruses, more specifically in *Chrysodeixis chalcites* and *Trichoplusia ni* SNPV (van Oers et al., 2004; Willis et al., 2005), where genes encoding putative photolyases are found. In other systems these enzymes repair DNA damage under the influence of blue light. This observation with baculoviruses was the rationale to investigate the biology and the ecology of this putative repair system further in this thesis.

Baculoviruses

Structure and taxonomy

Baculoviruses are highly pathogenic to the larval stages of certain arthropods, mainly insects, and often cause a fatal disease. The baculoviruses (Family *Baculoviridae*) form a group of large, enveloped DNA viruses isolated from over 600 host insect species, the majority from the orders Lepidoptera, Hymenoptera and Diptera (Blissard, 1996; Miller, 1997b; Thiem and Cheng, 2009). Baculoviruses have a circular, covalently closed, double-stranded DNA genome, ranging in size from 80 to 180 kilobase pair (kpb), that carry between 90 and 180 genes or open reading frames (ORFs) (Rohrmann, 2008a). The genomes are packaged in bacilliform-shaped nucleocapsids and the name "baculovirus" refers to the shape of the nucleocapsid (baculum = rod in Latin). As of May 2010, fifty-three baculovirus genome sequences have been deposited in the NCBI Genbank (http://www.ncbi.nlm.nih.gov/sites/entrez).

The virion(s) are occluded in a crystalline matrix, which is composed predominantly of a protein called polyhedrin or granulin to form the so-called occlusion bodies or OBs. OBs are stable and can survive within the host after assembly and during the disintegration of the host and persist most normal environmental conditions, thereby allowing the occluded virions to remain infectious over longer periods of time. Nucleopolyhedroviruses (NPVs) produce large polyhedron-shaped OBs called polyhedra which occlude many virions, while the granuloviruses (GVs) form a smaller OB containing granulin, containing a single virion. NPVs are either of the SNPV or MNPV phenotype, depending on whether the nucleocapsids are encapsulated singly (S) or as multiples (M) (Miller, 1997b; Rohrmann, 2008a; Slack and Arif, 2006) (Fig. 1A). The family *Baculoviridae* is taxonomically

divided into four genera: *Alphabaculovirus* and *Betabaculovirus,* which represent the NPVs and GVs, respectively, that infect lepidopteran insects. The baculoviruses that infect hymenopterans and dipterans are grouped in the *Gamma-* and *Deltabaculovirus,* respectively (Jehle et al., 2006a). Lepidopteran NPVs are further subdivided into group I and group II NPVs based on phylogenetic analysis (Herniou and Jehle, 2007), but the S or M signature of NPVs has no taxonomic meaning.
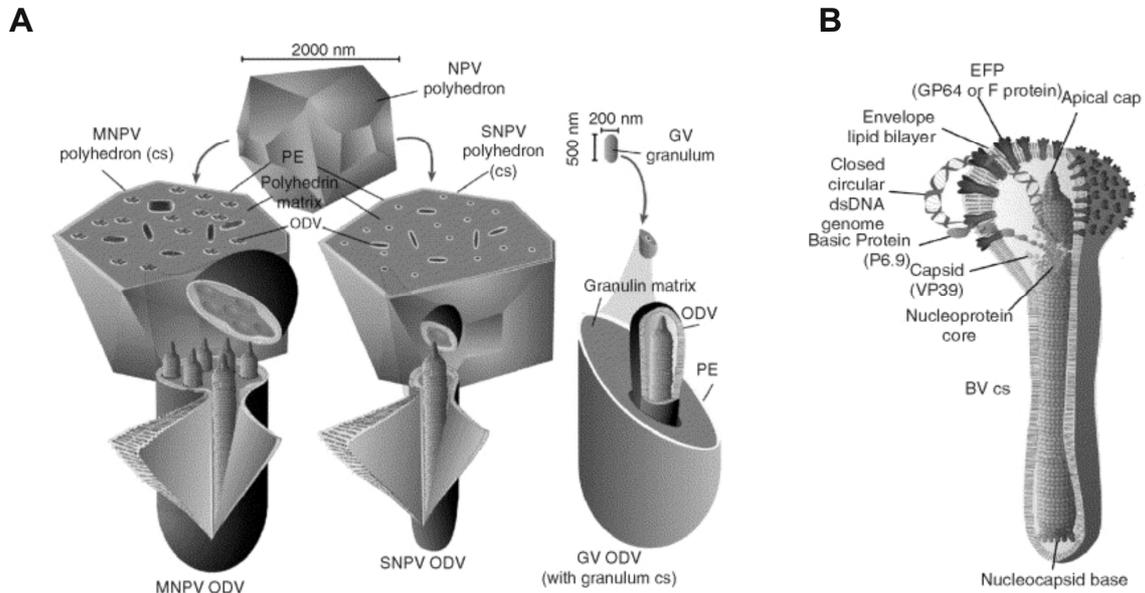

Figure 1. Structures of occlusion bodies (OBs) and occlussion derived viruses (ODVs) (A) and budded virus (BV) (B). Three major OB phenotypes are illustrated in the background. The OBs of Granuloviruses (GVs) are capsule-shaped containing only a single virion. Nucleopolyhedroviruses (NPVs) occluding multiple virions form large polyhedron-shaped OBs, which are larger than the OBs of GV. NPVs form ODVs with either multiple (MNPVs) or single (SNPVs) nucleopcapsids, as demonstrated in the foreground. In the infection cycle of a baculovirus, BVs are produced to cause a systemic infection in the infected insect. A dissected view of the structure of the BV is represented in (B). BVs contain single rod-shaped nucleocapsids. The major BV envelope fusion proteins and structural components are shown at the top and on the left of the virion (from Slack and Arif, 2007).

Two virion phenotypes are produced in the infection cycle of a typical baculovirus. Budded viruses (BVs) contain rod-shaped nucleocapsids singly (Fig. 1B). Occlusion derived virions (ODVs) may contain a single or multiple nucleocapsids, depending on whether the virus is a SNPV or GV, or MNPV. BVs and ODVs are genetically identical, but morphologically different entities. This is reflected in distinct protein and lipid composition of either phenotype. The envelope of BVs is derived from the plasma membrane at virion egress, while the membrane proteins of ODVs assemble *de novo* in the nucleus of infected cells (reviewed by Federici, 1997; Slack and Arif, 2006). BVs and ODVs play different roles in the infection cycle. ODVs initiate primary infections in midgut epithelial cells of susceptible hosts, while BVs spread the virus infection from cell to cell leading to systemic infection within one infected insect.

Infection cycle
OBs stabilize and protect the virions in nature against decay until susceptible insect hosts (larvae) are encountered, who take up OBs by the oral route. Upon ingestion by larvae, the OBs dissolve in the

alkaline environment of the midgut, releasing the ODVs. After fusion of the ODV envelope with the microvilli of midgut epithelial cells, the nucleocapsids are transported along the cytoskeleton and through the nuclear pores into the nucleus, where DNA expression, DNA replication and assembly of progeny virions occur (Federici, 1997) (Fig. 2A). In the early stage of infection, progeny virus buds through the basal lamina lining the midgut and connective tissue to initiate a systemic infection by infecting neighbouring cells and other susceptible tissues (Fig. 2B), such as haemocytes, fat body and trachea which, in turn, produce more BVs (Fig. 2C). In a later phase of the latter infection, the nucleocapsids are retained in the nucleus and assemble in a *de novo* produced envelop to produce ODVs. The ODVs are occluded in a matrix of polyhedrin or granulin protein to generate OBs. These OBs are ultimately released into the environment upon death and disintegration of the insect with the help of virus-encoded proteases and chtinases. OBs are responsible for virus transmission to the next host (Fig. 2D) (reviewed by Blissard and Rohrmann, 1990; Federici, 1997; Rohrmann, 2008b).
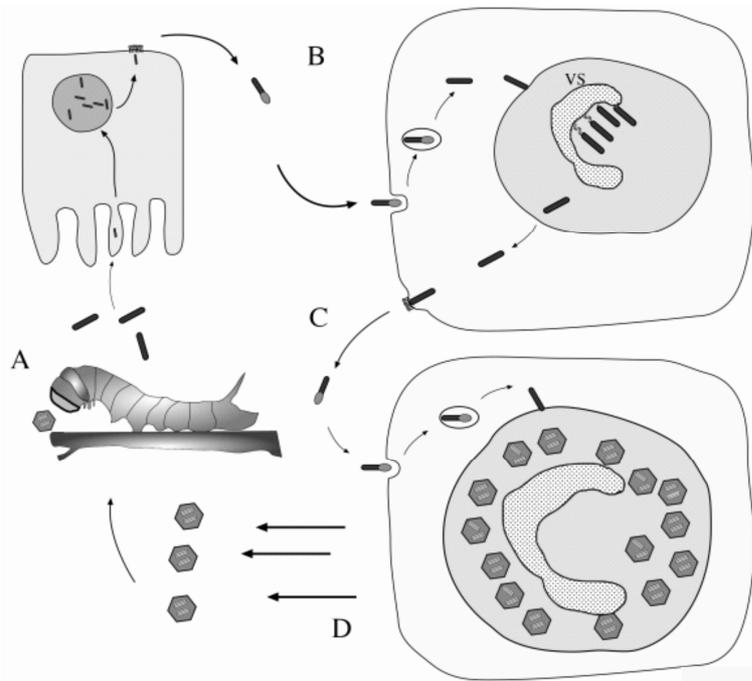
Figure 2. The infection cycle of a baculovirus. OBs are ingested by an insect, ODVs are released in the midgut and infect epithelial cells (A). The virions budded out of these cells initiate a systemic infection (B). More BVs are produced to spread the infection within the insect (C). ODVs are produced and released from the dead cells at a late stage of infection (D). The virogenic stroma (Vs) where DNA replication occurs is indicated (from Rohrmann, 2008)

Applications of baculoviruses

Baculovirus as vectors for gene expression and gene delivery

The Baculovirus Expression Vector System (BEVS) is widely used for the production of post-translationally modified, functional recombinant proteins in insect cells for scientific and medical purpose (Hitchman et al., 2009; Hu, 2005; Jarvis et al., 2009). The majority of commercial-available baculovirus vector systems is based on *Autographa californica* multiple NPV (AcMNPV), a virus with a genome of 133 kilobase pairs (Ayres et al., 1994), which efficiently infect a number of insect cell lines productively. The insect cell lines derived from *Spodoptera frugiperda* (Sf9 or Sf21) (Vaughn et al., 1977) and *Trichoplusia ni* (High Five™) cells (Granados et al., 1994), are commonly used for production of recombinant proteins with AcMNPV-based vectors. In this case the promoter of

polyhedrin, which is very strong, is used to drive the expression of a foreign gene. In cell culture polyhedrin and OBs are dispensable for virus replication and BVs are sufficient for infection. Literally, thousands of recombinant proteins have been produced in insect cells using this system to date (reviewed by Beljelarskaya, 2002; Hitchman et al., 2009; van Oers, 2006), among others for human and veterinary applications, for instance commercial vaccine is available against classical swine fever (CSF) (Depner et al., 2001) or is pending against influenza virus infection (FluBlok, Protein Sciences) (Cox and Hollister, 2009). A highly valuable application of this system is the production of virus-like particles (VLPs) and protein complexes (Noad and Roy, 2003) in insect cells. The first vaccine in the form of VLPs produced in insect cells for human use is on the market since 2008 (Cervarix, GSK). This vaccine consists of the L1 protein of human papilloma virus and is directed against cervical cancer (Stanley, 2003; Trimble and Frazer, 2009). This system has also been used to produce large quantities of infectious adeno-associated viruses (AAV). which are used as gene therapy vectors in clinical studies (Urabe et al., 2002).

In addition to the role of baculovirus as an insect cell protein expression system, its capability of efficiently transducing a wide variety of mammalian cells resulting in a novel baculovirus-based tool for *in vitro* and *in vivo* gene delivery. In contrast to many other commonly used viral vectors, baculoviruses are unable to replicate and produce infectious virus in mammalian cells. The viruses can be readily manipulated, accommodate large insertions of foreign DNA and show little observable cytopathic effects in mammalian cells (reviewed by Hu, 2006; Hu, 2008; Kost and Condreay, 2002). These characteristics are leading to increased interest and further development of this system for efficient gene delivery into mammalian cells *e.g.* for gene therapy purposes (Hu, 2006), cancer treatment (Song and Boyce, 2001; Stanbridge et al., 2003) or to deliver lentivirus vectors (Lesch et al., 2008). Modification towards tissue tropism (Räty et al., 2004) and inhibition of the mammalian complement system further contribute to the development of baculoviruses as gene therapy vector. (reviewed by Hu, 2006).

Baculovirus as biological control agents
The abundant use of conventional chemical pesticides to control pest insects has induced increased resistance of many insect species to those chemicals (Kirschbaum, 1985). The overdependence on these pesticides creates numerous unacceptable agricultural, environmental, and human health problems (Wood and Granados, 1991). Alternative bio-control approaches such as parasites, predators and microorganisms may provide good alternatives. Baculoviruses provide adequate virulence, host specificity and safety for the environment, which makes them attractive candidates for the biological control of insect pests. As a consequence, a number of baculoviruses have been developed and registered as commercial insect control products for the protection of crops, vegetables, forests, greenhouse flowers and pastures (Moscardi, 1999; Szewczyk et al., 2006). So far, the most widely applied commercial baculovirus product is based on *Anticarsia gemmatali* NPV and protects 650,000 to 1.7 million hectares/year in Brazil to control the velvetbean caterpillar, a pest of soybean (Moscardi, 1989). In North America and Europe, a granulovirus of *Cydia pomonella* (CpGV) is in use for the control of the codling moth on pear and apple on more than 100,000 hectares of orchards (Lacey et al., 2008). The cotton bollworm, *Helicoverpa armigera*, is a key pest of cotton and with the intensive use of chemical insecticides it has developed resistance against chemicals in many parts of the world. In China, *Helicoverpa armigera* NPV (HearNPV) is being used against this insect pest. To this aim, about 1,600 tons of formulated HearNPV was produced for instance in 2005 and used on an estimated 200,000 to 300,000 hectares of cotton (Sun and Peng, 2007).

The further commercial expansion of baculoviruses as bio-control agents is hampered by variable potency of viral batches, a relatively slow speed of killing compared to chemicals and limited product stability. Genetic engineering approaches have been used to enhance the pesticidal properties (*e.g.* virulence and killing speed) of baculoviruses (Inceoglu et al., 2006; Miller, 1995; Wood and Granados, 1991). One approach to improve the speed of action is the deletion of the viral ecdysteroid UDP-glucosyltransferase (*egt*) gene. The EGT enzyme blocks larval molting and prolongs host survival time to favour virus production. Deletion of the *egt* gene from AcMNPV genome resulted in a 30% improvement in speed of kill and a 40% reduction in food consumption compared with wild type virus (Cory et al., 2004; O'Reilly and Miller, 1991). In addition, several foreign genes have been proposed for insertion into viral genomes including insect-specific toxins, hormones and growth regulators (Miller, 1997b). Recombinant baculoviruses with inserted toxin genes provided an increased killing speed as well as lower larval feeding activity (McCutchen et al., 1991; Stewart et al., 1991).

Solar radiation is regarded as a major factor that negatively affects field performance of baculoviruses, by inactivating viral activity of occlusion bodies almost completely in less than 24 hours (Jaques, 1985). There is no difference between the solar inactivation patterns of wild-type and the recombinant baculoviruses described above with deleted *egt* gene or inserted toxin genes (Sun et al., 2004a). In order to extend the field persistence of baculoviruses, the UV sensitivity of the virus is reduced by adding UV screening agents (optical brighteners) to virus formulations such as uric acid, p-aminobenzoic acid, 2-hydroxy-4-methoxy-benzophenone, UVal and Tinopal DCS (Black et al., 1997; Moscardi, 1999). In addition to the above, carbon black has also been used as protective agent for a variety of baculovirus formulations, including *Helicoverpa zea* NPV (HzNPV), *Choristoneura fumiferana* NPV (CfNPV) and *Lymantria dispar* NPV (LdNPV) (Black et al., 1997). However, these UV protectant materials increase UV resistance to various extents, but none of these can protect baculoviruses in the field for more than 4 days (Black et al., 1997). Furthermore, UV screening agents may interfere with the intrinsic ability of baculoviruses to adhere to plant surfaces (Black et al., 1997). About half of the production costs are for UV protectants. So, UV inactivation is one of the major issues in the practical application of baculoviruses for insect control, except in protected crops in greenhouses.

Light-driven DNA repair by photolyase

UV-irradiated DNA lesions

Many organisms encounter exposure to UV radiation through sunlight. Solar UV radiation in the spectral region between 200-300 nm has harmful effects on living organisms such as growth delay, mutagenesis and even cell killing (Slaper et al., 1996; Vink and Roza, 2001). The most significant cellular target of UV is DNA. When DNA is exposed to radiation at wavelengths of around 260 nm, adjacent pyrimidines within the same DNA strand may become covalently linked to form cyclobutane pyrimidine (Pyr<>Pyr) dimers (CPD) (Setlow and Carrier, 1966) and pyrimidine–pyrimidone (6-4) photoproducts (Pyr [6-4] Pyr) (Rycyna and Alderfer, 1985) (Fig.3). *In vivo*, CPDs are more frequently formed than 6-4PPs at a ratio of about 3:1 (Essen and Klar, 2006). Both types of DNA lesions are cytotoxic as they block transcription, induce mutations and trigger apoptosis (Ljungman and Zhang, 1996; Nishigaki et al., 1998; Öztürk et al., 2008). Due to the fact that they cause mutations, pyrimidine dimers are also crucial players for the induction of skin cancer (Lima-Bessa and Menck, 2005).
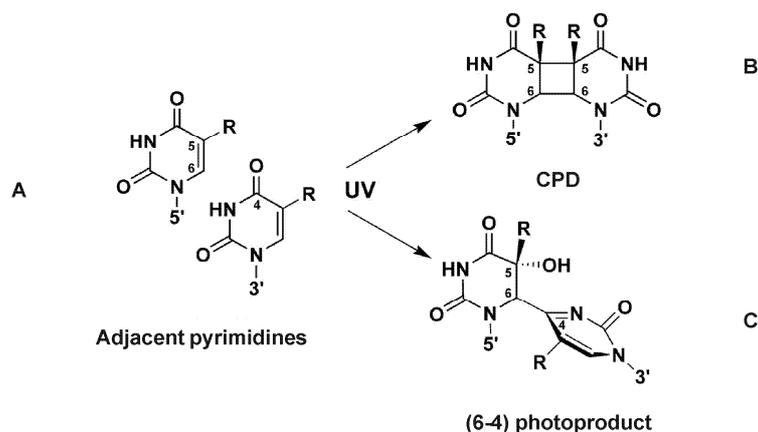
Figure 3. UV-induced DNA photoproducts. The two major lesions are formed between adjacent pyrimidine bases (A) in DNA induced by ultraviolet irradiation, including cyclobutane pyrimidine dimers (B) and pyrimidine–pyrimidone (6–4) photoproducts (C) (from Li et al., 2006)

Viruses can be inactivated by UV radiation in a similar way, the nucleic acid within the virus particle (DNA or RNA) plays a crucial role in the absorption of UV radiation and in virus inactivation (Rauth, 1965). The sensitivity to UV may correlate with the number of bases (molecular mass) in the viral genome, as the more target molecules, the more damages in genome may form at a given level of UV exposure (Lytle and Sagripanti, 2005; Rauth, 1965). In addition, nucleic acid in the single-stranded state is approximately ten times more sensitive to UV than in the double-stranded state (Rauth, 1965). The most common, lethal photoproducts induced by UV are pyrimidine dimers, particularly thymidine dimers (Friedberg et al., 1995). Since DNA but not RNA contains thymine, DNA-containing viruses are generally more sensitive to damage by UV than RNA-containing viruses (Kundu et al., 2004; Murphy and Gordon, 1981; Rauth, 1965).

DNA repair pathways
To maintain genetic integrity and to avoid toxic effects resulting from the dimeric pyrimidine products, several DNA repair mechanisms are known to be utilized by various organisms. These DNA repair systems can be distinguished with respect to their light dependency (Yasui and McCready, 1998). The light-independent repair pathways include nucleotide excision repair (NER) (Eker et al., 2009; Lehmann, 1995) and base excision repair (BER) (Dalhus et al., 2009; Kimura et al., 2004). NER is a highly conserved repair system present in almost all organisms. NER is an ATP-dependent, multi-step pathway that involves a protein complex in which the oligonucleotide to be repaired is excised from the DNA and the resulting single-strand gap is refilled eventually (Sancar, 1996; Van Houten, 1990). BER is another frequently used repair pathway involving multiple steps and several enzymes. In the system, the key enzyme is DNA glycosylase, which is responsible for recognizing and removing the pyrimidine dimers (Haseltine et al., 1980). Enzymatic photoreactivation is known to be a very powerful light-dependent repair mechanism for which blue light (400-500 nm) or near-UV light (320-400 nm) is required. Among the various repair systems, photoreactivation is the simplest and fastest pathway as it uses only visible light as energy source and involve a single enzyme, resulting in an energetically cheaper and more direct reaction than provided by other pathways (Weber, 2005). Photoreactivation occurs in all groups of organisms, except placental mammals, who have to rely on the NER pathway to repair pyrimidine dimers (Yasui et al., 1994).

DNA photolyase

Two photolyases are distinguished based on substrate specifity: CPD photolyase repair CPD lesions, whereas (6-4) photolyase restore (6-4)PPs in DNA. CPD photolyases are further divided into two classes, based on divergence of their amino acid sequence. Phylogenetic analysis suggested that both class I and class II CPD photolyases have a common ancestor but diverged in early evolution of archae (Yasui et al., 1994). Class I photolyases are found in many microbial organisms, while class II photolyases are encoded by eukaryotes and a few bacteria (O'Connor et al., 1996). Class II photolyases are also found in several pathogens, including some poxviruses (Bennett et al., 2003; Srinivasan et al., 2001) and insect parasitic *microsporidiae* (Slamovits and Keeling, 2004). Although placental mammals including humans lack photolyase (*phr*) genes, the human genome has two genes with partial sequence homology to photolyase genes. These genes (*cry*) encode cryptochromes that are involved in the biological clock, but do not have photoreactivating activity (Eker et al., 2009; van der Horst et al., 1999).

DNA photolyases are monomeric proteins with a molecular mass in the 45-66 kDa range and comprise 420-616 amino acid residues depending on the organism (Sancar, 1990). Photolyases always carry a flavin adenine dinucleotide (FAD) molecule as the catalytic co-factor. Besides FAD, many CPD photolyases also contain a light-harvesting co-factor, which is either 5,10-methenyltetrahydrofolate (MTHF) or an 8-hydroxy-7,8-didemethyl-5-deazaflavin (8-HDF). The light-harvesting co-factor captures near-UV or blue light photons (reviewed by Deisenhofer, 2000; Eker et al., 2009; Essen and Klar, 2006; Park et al., 1995; Sancar, 1990). More recently, flavin mononucleotide (FMN) was found as light-harvesting cofactor in the photolyase from the bacterium *Thermus thermophilus* HB8 (Ueda et al., 2005) and FAD was identified as a novel light-harvesting chromophore of photolyase from the archaeon, *Sulfolobus tokodaii* (Fujihashi et al., 2007).

For the primary structure of photolyase, two highly conserved regions of photolyases were revealed by the alignment of amino acid sequences. Two regions are predicted, respectively as CPD-DNA photolyase and FAD-binding domains, which are characteristic for all CPD photolyases (Sancar, 2003). Crystal structures have been solved for the class I photolyases from the bacteria *Escherichia coli* (Park et al., 1995) and *T. thermophilus* (Komori et al., 2001), and the cyanobacterium *Anacystis nidulans* (Tamada et al., 1997) at resolutions between 1.8 and 2.3 Å. These three photolyases show a common backbone structures: An N-terminal α/β domain (180-220 residues) composed of a five-stranded, parallel β-sheet, which is covered on both sides by α-helices. This N-domain is responsible for the binding of the light-harvesting chromophore. A C-terminal α-helical domain (240-280 residues) interacts with the catalytic cofactor FAD. The N- and C-terminal domains are connected via a long interdomain loop (reviewed by Essen and Klar, 2006; Müller and Carell, 2009; Weber, 2005).

The proposed reaction mechanism of the CPD I photolyase is presented in a commonly accepted model (Fig. 4). A prerequisite for the repair of the CPD lesion is the complete flipping-out of the pyrimidine dimer from the DNA helix into the active site of the CPD photolyase with which it forms a stable complex (Vande Berg and Sancar, 1998). After the photolyase-CPD substrate complex has formed the second co-factor absorbs a near UV/blue light photon and transfers the photon energy to the FAD chromophore, which is thereby converted from FADH$^-$ to the excited form *FADH$^-$. This *FADH$^-$ transfers an electron to the CPD lesion leading to cleavage of the cyclobutane ring. The CPD splits into two pyrimidines and an electron is transferred back to restore the functional FADH$^-$ form. Finally, the repaired DNA is released from the DNA photolyase and the two DNA strands reanneal. However, details of several reaction steps in this catalytic cycle are still hypothetic (Essen, 2006; Sancar, 2003; Weber, 2005).
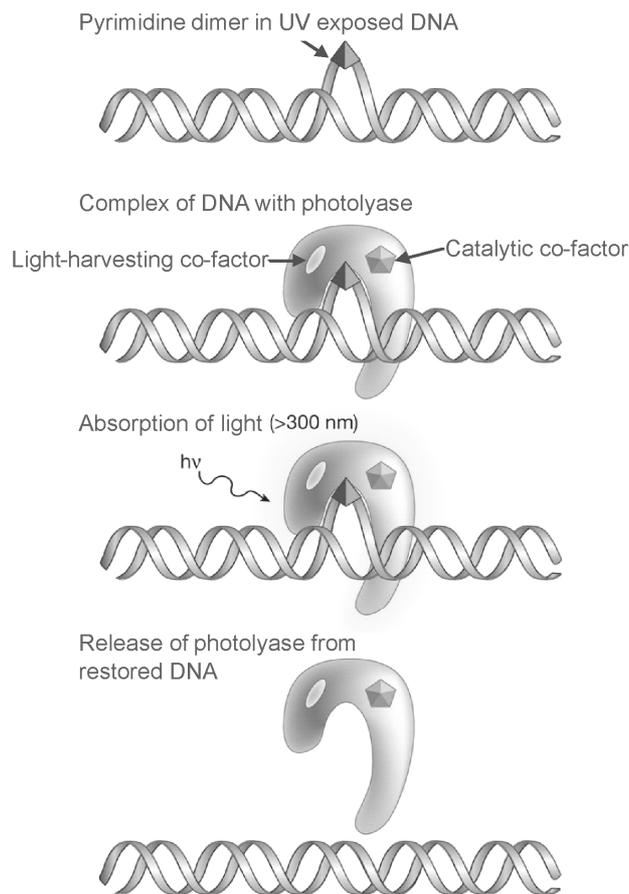
Figure 4. The proposed reaction mechanism of photoreactivation. The pyrimidine dimers are recognized and bound by a photolyase. Upon absorption of light at wavelengths >300 nm by the light-harvesting chromophore, energy is transferred to the catalytic co-factor (FAD), which transfers an electron to the pyrimidine dimer and restores them to the native conformation (adapted from Friedberg, 2003).

DNA photolyases in baculovirus

Class II CPD photolyases have been discovered in several chordopoxpoxviruses, including the avipoxvius Fowlpox virus (FWPV) (Afonso et al., 2000), and the leporipoxviruses Myxomavirus (MYXV) and Shope fibroma virus (SFV) (Cameron et al., 1999; Willer et al., 1999). Such photolyases wee also discovered in the insect poxviruses *Melanoplus sanguinipes* entomopoxvirus (MSEV) (Afonso et al., 1999) and *Amsacta moorei* entomopoxvirus (AMEV) (Bawden et al., 2000). The photolyase encoded by FWPV was shown to utilize light to repair lesions in the viral genome, thereby restoring the infectivity of UV-damaged virus and promote the survival of FWPV in the environment (Srinivasan et al., 2001; Srinivasan and Tripathy, 2005).

In 2004, two genes for class II CPD photolyase, designated *phr1* and *phr2*, were identified in a Dutch isolate of the baculovirus C*hrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) (van Oers et al., 2005; van Oers et al., 2004). Two domains, a CPD-DNA photolyase domain and an FAD binding domain, which are characteristic for class II CPD photolyases, are conserved in the photolyases of ChchNPV. A homologous gene is present in the genome of *Trichoplusia ni* (Tn) SNPV (Willis et al., 2005). The photolyase proteins encoded by ChchNPV, Cc-PHR1 and Cc-PHR2, share 45% identity at the amino acid level. Tn-PHR is most closely related to Cc-PHR1 with which it shares 78% amino acid identity. Phylogenetic analysis based on the amino acid sequences of 29 baculovirus core genes

has shown that ChchNPV and TnSNPV are closely related and form a separate clade in group II NPVs (Herniou and Jehle, 2007). ChchNPV and TnSNPV, both infect lepidopteran insects in the subfamily Plusiinae of the family Noctuidae (van Oers et al., 2004). Recently, a single CPD photolyase gene was also found in the genome of *Spodoptera litura* granulovirus (SpliGV) (Wang et al., 2008). In addition, two small photolyase ORFs separated by 1 bp were recently reported in the genome of *Clanis bilineata* (Clbi) NPV (Zhu et al., 2009). The ClbiNPV *phrs* correspond to the CPD-DNA photolyase and FAD-binding domains of photolyases, respectively.

The transcriptional activity of *Cc-phr2* was demonstrated by RT-PCR in ChchNPV-infected *C. chalcites* larvae and in infected *T. ni* High Five cells (van Oers et al., 2004). In the sequence upstream of the *Cc-phr2* ORF, three consensus GATA motifs were found (van Oers et al., 2004) while motifs characteristic for early (CAGT) or late (TAAG) baculovirus transcription initiation (Blissard and Rohrmann, 1990) are absent. GATA motifs are known to be recognized by members of the GATA family of zinc-finger transcription factors. GATA motifs have also been found in the *Orgya pseudotsugata* (Op) MNPV *gp64* gene, whose early promoter is activated by host transcription factor binding to these motifs (Kogan and Blissard, 1994). Tn-PHR was also predicted to be an early gene product based on two putative overlapping CAGT early promoter sequences in the *tn-phr* gene (Willis et al., 2005). Whether Cc-PHRs and Tn-PHR are true early gene products remains to be determined.

The activity of the two ChchNPV PHR proteins has been examined in a DNA repair deficient *E. coli* strain. The complementation assay showed that *Cc-phr2* encodes an active CPD photolyase; Cc-PHR1 could not complement a DNA repair deficient *E. coli* strain and this has been explained by the fact that a conserved tryptophan which may be involved in electron transfer (Aubert et al., 2000) is absent in PHR1 (van Oers et al., 2008). Purified recombinant Cc-PHR2 also repaired CPD dimers *in vitro*. Spectral measurements demonstrated the presence of FAD in Cc-PHR2 photolyase. Reconstitution experiments suggested that Cc-PHR2 uses a 8-HDF as second co-factor (van Oers et al., 2008).

Outline of the thesis

Baculoviral CPD photolyases have a potential function in repairing UV-induced damage in viral DNA in a light-dependant manner and form a rather unique feature among baculoviruses. Only three baculoviruses have been reported to possess full length DNA photolyase genes in the now 53 determined baculovirus genome sequences. The baculovirus photolyase Cc-PHR2 has been shown to possess photoreactivating ability in a heterogeneous system. So, the question is what role these enzymes play in the infection process. Is it at entry to rescue any defect of the incoming virus? Or is it during replication of the virus? Limited knowledge is also available about the possible existence of CPD photolyase genes in other baculoviruses and their potential role during the infection cycle of a baculovirus. It is also not known whether photolyases provide a baculovirus with an ecological benefit. In this thesis an attempt is made to answer the above questions, with the CPD photolyases encoded by ChchNPV as a model.

ChchNPV and TnSNPV are related group II NPVs that infect insects in the Plusiinae subfamily. Since they both carry one or two photolyase genes, the question is raised in **Chapter 2** whether photolyase genes are conserved in all group II NPVs infecting noctuid insects in the plusiine subfamily and whether or not all baculovirus photolyase genes have a common ancestor. To that aim, a PCR-based test is developed and a survey is performed to study the presence of *phr* genes in six

additional Plusiinae-infecting NPV isolates. PHR proteins need to be targeted to the nucleus, where baculoviruses replicate and assemble, to be able to repair DNA damage in a baculovirus genome and these proteins should have the ability to bind DNA. Therefore the intercellular localization of the two ChchNPV PHR proteins in insect cells is studied in a transient expression assay in **Chapter 3** and the effect of a baculovirus infection on the localization of these proteins is investigated. If baculoviruses need DNA repair at entry into midgut epithelial cells prior to replication, it is assumed the PHR proteins will be present in the ODV, as is the case with (entomo)poxviruses. In **Chapter 4**, the proteome of ChchNPV ODV virions is analysed to investigate whether PHRs are present in these virus particles. HearNPV has been developed as commercial biopesticide to control the cotton boll worm in China. If PHR proteins really contribute to the UV resistance of baculoviruses, it is surmised that addition of *phr* genes to baculoviruses lacking these enzymes would reduce their UV sensitivity. In order to improve the resistance of HearNPV to UV light, a recombinant HearNPV harboring *Cc-phr2* is constructed and tested in *H. armigera* larvae after UV irradiation. The results are described in **Chapter 5**. To be able to study ChchNPV DNA photolyases in further detail in the future, for instance by making ChchNPV deletion mutants, a cell line susceptible for ChchNPV is highly desirable. Therefore, a new cell line derived from *C. chalcites* embryos is established and characterized in **Chapter 6**. The overall results are summarized and discussed in **Chapter 7** as well as perspectives for future research and application.

Chapter 2

Conservation of DNA photolyase genes in group II nucleopolyhedroviruses infecting plusiine insects

Abstract

DNA photolyase genes (*phr*) encode photoreactive enzymes, which are involved in the repair of UV-damaged DNA. Cyclobutane pyrimidine dimer (CPD) specific photolyase genes are present in nucleopolyhedroviruses isolated from *Chrysodeixis chalcites* (ChchNPV) and *Trichoplusia ni* (TnSNPV), insects belonging to the Plusiinae (Noctuidae). To better understand the occurrence and evolution of these genes in baculoviruses, we investigated their possible conservation in other group II NPVs, which infect plusiine insects. A PCR based strategy using degenerate *phr*-specific primers was designed to detect and analyze possible photolyase genes. Six additional Plusiinae-infecting NPVs were analyzed and all, except *Thysanoplusia oricalcea* NPV A28-1, which is a group I NPV, contained one or more *phr*-like sequences. Phylogenetic analysis revealed that all photolyase genes of the tested Plusiinae-infecting baculoviruses group in a single clade, separated into three subgroups. The phylogeny of the polyhedrin sequences of these viruses confirmed that the analyzed viruses also formed a single clade in group II NPVs. We hypothesize that all plusiine group II NPVs contain one or more photolyase genes and that these have a common ancestor.

Keywords: DNA photolyase, *phr*, DNA repair, phylogeny, baculoviruses, Plusiinae

1. Introduction

Many organisms encounter DNA damage due to exposure to natural UV irradiation as present in sunlight. This damage consists of two types of major lesions: cyclobutane pyrimidine (Pyr<>Pyr) dimers (CPD) (Setlow and Carrier, 1966) and (6-4) photoproducts (Pyr [6-4] Pyr) (Rycyna and Alderfer, 1985). CPD lesions account for the majority of the DNA damage in *vivo* and are cytotoxic by interfering with transcription and inducing mutagenesis (Sancar, 1994); (Vink and Roza, 2001). In many organisms, except placental mammals, CPDs as well as 6-4 photoproducts can be repaired by enzymatic photoreactivation (Hearst, 1995). CPD photolyases specifically catalyze the photoreactivation of CPDs using the blue region of the visible light spectrum (Essen and Klar, 2006).

Two classes of CPD photolyases, class I and class II, have been distinguished based on divergence in amino acids sequence. The phylogenetic analysis suggested that both type I and type II photolyases have a common ancestor but diverged very early in evolution (Yasui et al., 1994). (Deisenhofer, 2000; Sancar, 2003)A class I photolyase, from *Escherichia coli*, was the first to be cloned and biochemically characterized (Sancar and Rupert, 1978; Sancar and Sancar, 1984) and the first for which the crystal structure was determined (Park et al., 1995). Much less is known about class II photolyases. CPD photolyases are monomeric proteins of 450-550 amino acids, which bind to CPD-containing-DNAs in a light-independent manner. These enzymes use non-covalently bound chromophores or co-factors. One of these cofactors is flavine adenine dinucleotide (FAD); the other is either 5, 10-methenyltetrahydrofolate (MTHF) or 8-hydroxy-7, 8-didemethyl-5-deazaflavin (8-HDF). The second chromophore captures blue light photons and the energy from these photons is transferred to FAD. An electron is then transferred from the excited FAD to the cyclobutane pyrimidine dimer, which is then converted in pyrimidine monomers (reviewed by Deisenhofer, 2000; Essen and Klar, 2006; Park et al., 1995).

CPD photolyases are present in all groups of organisms except placental mammals. The latter have to rely on the nucleotide excision repair system to repair UV damage (Schul et al., 2002). However, several poxviruses have been discovered to encode class II CPD DNA photolyases, including Chordopoxviruses such as the Avipoxvirus fowlpox virus (Afonso et al., 2000), and the Leporipoxviruses, myxomavirus (MYXV) and shope fibroma virus (SFV) (Cameron et al., 1999; Willer et al., 1999). The insect poxviruses *Melanoplus sanguinipes* entomopoxvirus (MsEV) (Afonso et al., 1999) and *Amsacta moorei* entomopoxvirus (AmEV) (Bawden et al., 2000) also contain a class II CPD DNA photolyase gene. More recently, two class II CPD photolyase genes, designated as *phr1* and *phr2*, were identified in a Dutch isolate of the baculovirus *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) (van Oers et al., 2005; van Oers et al., 2004). A single photolyase gene is present in the genome of *Trichoplusia ni* (Tn) SNPV (Willis et al., 2005). Amino acid sequence alignment showed 45% amino acid identity between Chch-PHR1 and Chch-PHR2 photolyases. Tn-PHR is most closely related to Chch-PHR1 with which it shares 78% amino acid identity. Phylogenetic analysis based on polyhedrin, the major NPV occlusion body protein, has shown that ChchNPV and TnSNPV form a separate clade (van Oers et al., 2004) in group II NPVs (reviewed by Herniou and Jehle, 2007). Both these viruses infect noctuid Lepidoptera of the subfamily Plusiinae. To our knowledge group I NPVs infecting Plusiinae do not contain photolyase genes (van Oers and Vlak, 2007). Recently, a full length DNA photolyase ORF was also found in the genome of *Spodoptera litura* granulovirus (SpliGV) (Wang et al., 2008), thus from a non-plusiine noctuid. A majority of the more than 40 sequenced baculovirus genomes, however, do not contain photolyase genes.

In this paper we investigated whether photolyase genes are conserved in other group II NPVs, which infect insects of the Plusiinae subfamily. Therefore, a PCR-based test and survey were developed to study the presence of *phr* genes in six additional Plusiinae-infecting NPV isolates. A phylogenetic tree was constructed to see whether baculovirus photolyase genes may have a common ancestor.

2. Materials and methods

2.1. Viruses and Viral DNA isolation

Polyhedra or DNA of six isolates from Plusiinae-infecting NPVs (family *Baculoviridae)* were collected to detect the presence of photolyase genes: *Plusia acuta* (Plac) NPV (No.115, BBA Federal Biological Research Center for Agriculture and Forestry, Institute for Biological Control, Germany), *Thysanoplusia orichalcea* (Thor) NPV B9 isolate from Dr Elisabeth Herniou, a ThorNPV A28-1 isolate originally from Dr Xiaowen Cheng (Cheng et al., 2005), a Spanish ChchNPV isolate (ChchNPV-SP) provided by Dr Delia Muñoz and *Pseudoplusia includens* (Psin) NPV (isolates from Los Angeles (LA) and Guatemala (GT)), obtained from Dr James Fuxa. The Dutch ChchNPV isolate (now named ChChNPV-NL) was described before (van Oers et al., 2004).

To isolate viral DNA from polyhedra, occlusion-derived virus particles (ODVs) were released by dissolving polyhedra in 0.1 M Na_2CO_3 for 10 min at 37ºC. The pH was neutralized with 1/6 volume 0.2 M Tris/HCL, pH 8.0. After centrifugation for 8 min at 6800 rpm in an Eppendorf centrifuge, the supernatant containing the ODVs was collected. Virus DNA was isolated from those ODVs by following the protocol for tissue DNA purification of the DNeasy tissue kit (Qiagen). The isolated viral DNA was stored at -20 ºC until further use.

2.2. Primer design and PCR amplification

To obtain partial sequences of photolyase genes, a set of degenerate *phr*-specific primers, PHR-DEG-F (5'-TGTAAAACGACGGCCAGTCARGTNGAYGCNCAYAAYGT-3') and PHR-DEG-R (5'-CAGGAAACAGCTATGACCDATYTTYTTNGCCCARTA-3'), was designed based on the two regions with the highest degree of conservation in class II CPD photolyases (QVDAHN and YWAKKI). These primers contained M13F or M13R primer tails and were used to carry out PCR. The length of the amplicon was expected to be about 780 bp. ODV-derived DNA of the various baculoviruses mentioned above was used as templates. A partial sequence of polyhedrin was also amplified by PCR using degenerate primers POLH-JOHF (5'-GTAAAACGACGCCCAGNRCNGARGAYCCNT-3') and POLH-JOHR (5'GGATAACAATTTCACACAGGDGGNGCRAAY-3'), as described (Jehle et al., 2006b). The amplified sequence for the polyhedrin genes was about 510 bp long, covering 2/3 of the complete polyhedrin open reading frame (Jehle et al., 2006b). The amplification reactions of 50 μl contained Phusion HF (or GC) Buffer (FINNZYMES), 15 pmol of each primer, 1 U Phusion DNA polymerase (FINNZYMES), 0.2 mM dNTPs (Invitrogen) and 10-50 ng of template DNA. The amplification conditions for photolyase sequences were as follows: an initial denaturation step at 98ºC for 3 min, followed by 35 cycles of 98ºC for 30 sec, 58 ºC for 30 sec, 72 ºC for 1 min, and a final extension step at 72ºC for 7 min. Polyhedrin sequences were amplified with an annealing temperature of 55ºC.

Table 1
Overview of the photolyase and polyhedrin (granulin) sequences used for phylogenetic analysis
A. Photolyase sequences

Organism Name	Accession No. / source
Bacillus cereus	AE016877
Drosophila pseudooscura	CM000071
Drosophila melanogaster	S73530
Carassius auratus	D11391
Danio rerio	BC054710
Bombyx mori	Silkworm.genomics.org.cn; Bmb009672
Melanoplus sanguinipes entomopoxvirus (MsEV)	AF063866
Shope fibroma virus (SFV)	AF170722
Amsacta moorei entomopoxvirus (AmEV)	AF250284
Myxoma virus (MYXV)	AF170726
Spodoptera litura GV (SpliGV)	NC_009503
Chrysodeixis chalcites NPV (ChchNPV) (NL)	NC_007151
Trichoplusia ni SNPV (TnSNPV-Ca)	NC_007383

B. Polyhedrin and granulin sequences

Organism Name	Abbreviation	Accession No. *polh/gran*
Adhoxophyes honmai NPV	AdhoNPV	AP006270
Anagrapha falcifera NPV	AnfaNPV	AY706686
Bombyx mori NPV	BmNPV	DQ231336
Buzura suppresaria NPV	BusuNPV	X70844
Chrysodeixis chalcites NPV	ChchNPV	YP249605
Clanis bilineata NPV	ClbiNPV	YP717539
Helicoverpa armigera NPV G4	HearNPV-G4	AF271059
Hemerocampa vetusta NPV	HeveNPV	AY706699
Leucania separata NPV	LeseNPV	U30302
Malacosoma americanum NPV	MaamNPV	AY706704
Mamestra brassicae MNPV	MbMNPV	AY706706
Mamestra configurata NPV	MacoNPV	AY126275
Malacosoma disstria NPV	MadiNPV	U61732
Orgyia pseudotsugata SNPV	OpSNPV	M32433
Orgyia pseudotsugata MNPV	OpMNPV	U75930
Orgyia anartoides NPV	OranNPV	AF068188
Panolis flammea NPV	PaflNPV	D00437
Plusia acuta NPV	PlacNPV	AY706712
Plusia orichalcea NPV	PlorNPV	AF019882
Rachiplusia ou MNPV	RoMNPV	AY145471
Spodoptera exigua MNPV	SeMNPV	AF169823
Spodoptera frugiperda NPV	SfMNPV	J04333
Thysanoplusia orichalcea NPV	ThorNPV_B9	Courtesy of E. Herniou
Thysanoplusia orichalcea NPV	ThorNPV A28-1	AY706719
Trichoplusia ni SNPV	TnSNPV-Ca	YP308889
Trichoplusia ni SNPV	TnSNPV-SA	AF093405
Cydia pomonella GV	CpGV	AY706670
Spodoptera litura GV	SpliGV	YP001256952

2.3. Validation of PCR method

To examine whether the PCR method with degenerate primers can indeed amplify different photolyase genes from baculoviral DNA, the complete open reading frames (ORF) of ChchNPV *phr1* and *phr2* were cloned into pIZ/His-V5 (Invitrogen), resulting in pIZ-*phr1* and pIZ-*phr2*. Cloned ChchNPV *phr1* and *phr2* ORFs, as well as ChchNPV viral DNA, were targeted as template, respectively, in a PCR with the degenerate PHR-DEG-F and PHR-DEG-R primers. The PCR products were purified with the GFX^{TM} PCR DNA and gel band purification kit (GE Healthcare) and digested with *Msc*I (Invitrogen) to discriminate the two ChchNPV *phr* ORFs. The reaction products were examined by electrophoresis in a 2% agarose gel stained with ethidium bromide.

2.4. DNA cloning and sequencing

The amplicons of photolyase were examined by agarose gel electrophoresis (not shown) and purified with GFX^{TM} PCR DNA and gel band purification kit (GE Healthcare). The amplified DNA was sequenced by automatic sequencing (MWG Biotech AG, Germany). When complicated sequence profiles were observed, indicative of the presence of more than one *phr* gene, the purified photolyase fragments were cloned in Clonejet 1.2 vector (Fermentas). In that case, three or more DNA clones were analyzed to determine the sequence of each *phr*-fragment in a particular viral genome. A partial nucleotide sequence of the polyhedrin gene was also determined directly on PCR products or on cloned inserts. The sequences were assembled and analyzed with DNAstar and Vector NTI software.

2.5. Phylogenetic analysis

Translated Blast (BlastX) searches were performed in GenBank of the National Center for Biotechnology Information (NCBI) to predict the closest homologues of the amplified sequences. Other photolyase genes were also retrieved from GenBank (Table 1A). Multiple alignments were performed with CLUSTALW (MEGA version 4 package) based on nucleotide and amino acid sequences separately. A Neighbor-joining (NJ) phylogenetic tree of the photolyases was constructed based on the nucleotide sequences by employing the Maximum Composite Likelihood model using MEGA 4 software. The robustness of the plylogenetic tree was tested by bootstrap analysis of 500 replications. Additionally, a phylogenetic tree derived from 29 NPV polyhedrins was generated in a similar way, using *Cydia pomonella* GV and *Spodoptera litura* GV as outgroup. The identities of nucleotide and amino acid sequences among baculovirus photolyases were computed with GeneDoc 2.0.

3. Results

3.1. PCR method validation for phr detection

Cloned ChchNPV *phr1* and *phr2* genes, as well as ChchNPV viral DNA were used as templates for PCR. Degenerate primers were used to test whether the designed PCR method can amplify different photolyase genes from a single batch of viral DNA. Digestion of the amplified products with *Msc*I easily discriminated the two *phr* ORFs in ChchNPV-NL (Fig. 1). With the cloned *phr1* gene as template, restriction fragments of 280 and 460 bp were obtained from the PCR product upon cleavage with *Msc*I (lane 1). Three fragments of 97, 280 and 351 bp, respectively, were derived from the amplified *phr2* sequence (lane 2). The sizes obtained were in line with what was expected from the published ChchNPV genome data (van Oers et al., 2005). When a PCR was performed with

ChchNPV-NL viral DNA as template (lane 3) four fragments were obtained after *Msc*I digestion corresponding to all fragment sizes obtained from *phr1* and *phr2,* individually. This result demonstrated that different photolyase genes can be amplified by PCR, even in a single reaction by using this degenerate primer set to detect *phr* genes.
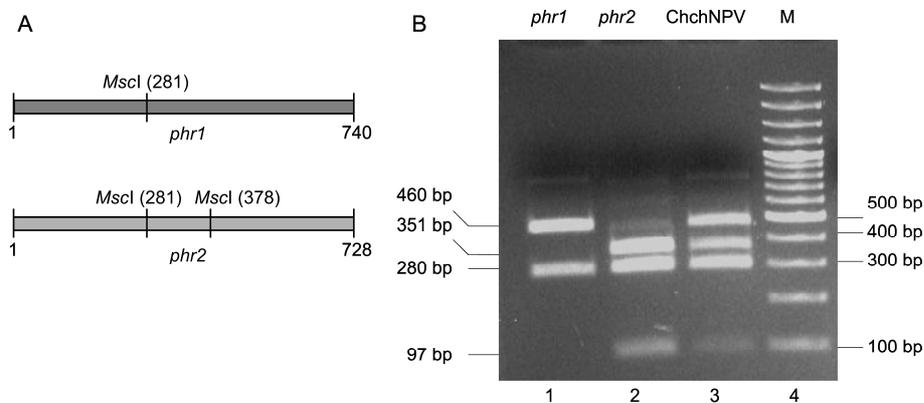

Figure 1. Validation of the PCR method. (A) Physical map of individually amplified *phr* sequences for *phr1* and *phr2* upon digestion with *Msc*I. (B) *Msc*I digestion of *phr* sequences amplified from cloned ChchNPV *phr1* (lane 1) or *phr2* (lane 2) genes *or* from ODV-derived ChchNPV DNA (lane 3). The generated fragments were separated in a 2% agarose gel stained with ethidium bromide. A 100 bp DNA ladder plus (lane 4) (GeneRuler, Fermentas) is indicated on the right in bp.

3.2. Detection of novel photolyase sequences

With the PCR method validated above PCR products were obtained for all analyzed viruses, except for ThorNPV A28-1. Translated Blast (BlastX) searches on the sequences obtained revealed that the Spanish ChchNPV isolate (ChchNPV-SP), PsinNPV-GT and PlacNPV contained two divergent *phr* genes. These *phr* genes showed high amino acid identities to their closest homologues in the Dutch ChchNPV isolated (ChchNPV-NL PHR1 and PHR2) (Table 2). For the other NPVs analyzed, only a single *phr* sequence was observed, which is was most similar to *phr1* of ChchNPV-NL, except for the *phr* sequence amplified from ThorNPV-B9, which is more similar to ChchNPV-NL *phr2*. The alignment also demonstrated that both PHRs from ChchNPV-SP, PlacNPV and ChchNPV-NL share 100% identity on the amino acid level (Table 2; Fig. 2.). In contrast to these plusiine photolyase genes, the SpliGV photolyase shows relatively low amino acid and nucleotide identities to both *phr1* and *phr2* of the Dutch ChchNPV isolate.

3.3. Phylogenetic relationship between phr genes

To study the evolutionary relationship of these baculovirus photolyases, they were compared with other photolyase nucleotide sequences selected in GenBank including those derived from vertebrate viruses, entomopoxviruses and insects species (Table 1A). The *phr* gene of the bacterium *Bacillus cereus* was used as out-group enabling rooting of the tree (Fig. 3A).

The results revealed that the photolyases of the Plusiinae-infecting baculoviruses (ThorNPV-B9, ChchNPV-NL, ChchNPV-SP, PsinNPV-GT/LA, PlacNPV and TnSNPV) form a clade distinct from a clade containing the dipteran insects (*Drosophila melanogaster* and *D. pseudoobscura*) and the silkworm *Bombyx mori,* and from one comprising the animal-poxviruses (MYXV and SFV) and SpliGV. The clade containing the fishes (*Danio rero* and *Carassius auratus*) and the one comprising the entomopoxviruses (MsEV and AmEV) show a relatively longer distance to the Plusiine

photolyases. Interestingly, the photolyase of SpliGV does not fall into the same clade as the other baculovirus photolyases do, but is more related to that of animal-infecting poxviruses. Within the clade of Plusiinae-infecting group II NPVs, the *phr1* genes of ChchNPV-SP1, PsinNPV-GT1, PsinNPV-LA and PlacNPV-1 form a group with *phr*1 of ChchNPV-NL and *phr* of TnSNPV. The *phr2* genes of ChchNPV-NL, ChchNPV-SP, and PlacNPV form a separate group. The topology of the tree also indicated that the photolyases of ThorNPV-B9 and PsinNPV-GT2 are distant from the sequences in both the *phr1* and *phr2* clades.

Phylogenetic analysis of polyhedrin and granulin nucleotide sequences derived from 29 NPVs, mainly derived from group II, and 2 GVs (Table 1B) was performed to demonstrate the evolutionary position of the Plusiinae-infecting NPVs used in this assay. The topology shows that the viruses analyzed here for photolyase genes clearly classify among group II NPVs and form a distinct clade within this group (Fig. 3B). The exception is ThorNPV A28-1, which did not amplify a photolyase PCR product and is a group I plusiine NPV, together with *Rachiplusia ou* (Ro) MNPV and *Anagrapha falcifera* (Anfa) MNPV.
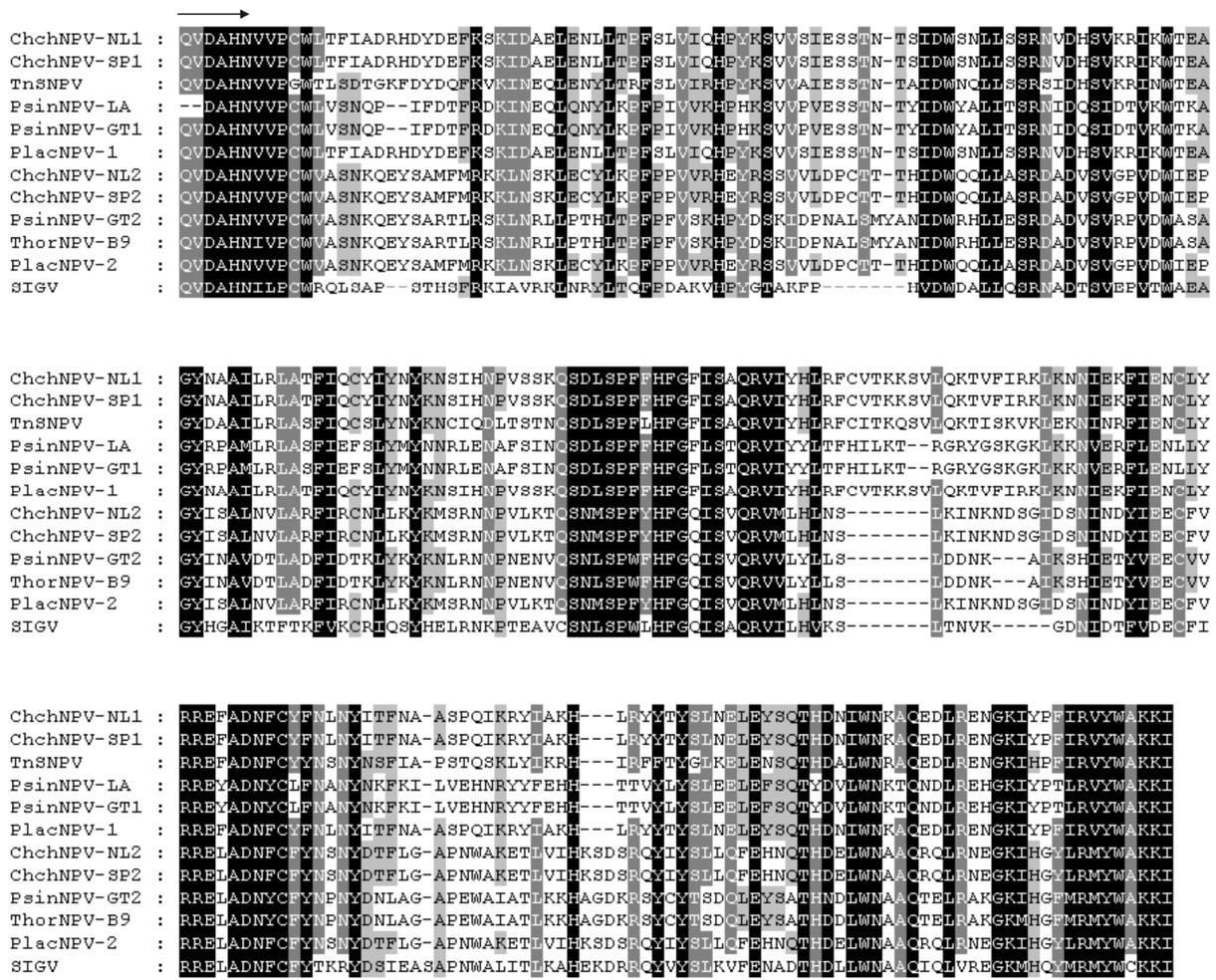

Figure 2. Alignment of partial CPD DNA photolyases in baculoviruses. Three shading levels were used: black for 100% similarity, dark gray for a minimal of 80% and light gray for at least 60% similarity. Two regions of PHR degenerate primers were marked with arrow heads.

Table 2

Pairwise comparisons of nucleotide and amino acid sequences for photolyases in baculoviruses*.

Class		ChchNPV NL1	ChchNPV SP1	TnSNPV	PsinNPV LA	PsinNPV GT1	PlacNPV 1	ChchNPV NL2	ChchNPV SP2	PlacNPV 2	PsinNPV GT2	ThorNPV B9	SpliGV
phrA	ChchNPV NL1	-	98%	78%	67%	67%	98%	54%	53%	53%	48%	48%	42%
	ChchNPV SP1	100%	-	78%	67%	67%	97%	53%	53%	53%	49%	49%	42%
	TnSNPV	85%	85%	-	67%	68%	78%	53%	53%	53%	49%	49%	44%
	PsinNPV LA	70%	70%	74%	-	99%	67%	50%	50%	50%	48%	48%	41%
	PsinNPV GT1	71%	71%	74%	99%	-	67%	50%	50%	50%	48%	48%	41%
	PlacNPV 1	100%	100%	85%	70%	71%	-	52%	53%	53%	48%	48%	42%
phrB	ChchNPV NL2	62%	62%	62%	58%	58%	62%	-	98%	98%	59%	59%	49%
	ChchNPV SP2	62%	62%	62%	58%	58%	62%	100%	-	99%	59%	59%	50%
	PlacNPV 2	62%	62%	62%	58%	58%	62%	100%	100%	-	59%	59%	50%
phrC	PsinNPV GT2	61%	61%	58%	56%	57%	61%	72%	72%	72%	-	98%	55%
	ThorNPV B9	61%	61%	58%	56%	57%	61%	72%	72%	72%	100%	-	56%
phrD	SpliGV	52%	52%	52%	50%	51%	52%	60%	60%	60%	64%	64%	-

*The percentage of nucleotide identity is given above and amino acid identity is shown below the diagonal. Identities were calculated in the range of the amplified segment only.

4. Discussion and conclusions

Partial coding sequences (with a maximum of 780 bp) of putative DNA photolyase genes were obtained from five out of six Plusiinae-infecting baculoviruses using a PCR method with a degenerate primer set. Only ThorNPV isolate A28-1 failed to produce a PCR product with this primer set. *Msc*I digestion of amplified photolyase sequences from ChchNPV-NL showed that the designed PCR method is robust enough to amplify different variants of photolyase genes even when present in the genome of a single virus. In the current report this was shown for ChchNPV-SP, PlacNPV and PsinNPV-GT, where also two different *phr* genes were found. Whether these reflect two *phr* genes present in the same genome, or whether two genomic variants exist in the same isolate can not be concluded from our data. For the other NPVs we observed only a single photolyase sequence..

Novel photolyase homologues derived from the plusiine baculoviruses tested, belong based on homology searches roughly into two types and are most similar to either the ChChNPV-NL *phr-1* and TnSNPV *phr* on the one hand or to ChchNPV-NL *phr-2* on the other; (van Oers et al., 2005; Willis et al., 2005). Phylogenetic analysis (Fig. 3A) showed that the photolyases of Plusiinae-infecting baculoviruses are separated from other photolyases to form one clade. This strongly suggests that all these photolyase genes originated from a common ancestor. The branch topology further suggested that the clade with PsinNPV-GT2 and ThorNPV-B9 is outside both the *phr1* and *phr2* group. The phylogenetic tree further showed that the photolyase of SpliGV is not closely related to the clade of Plusiinae photolyases. Its position in the tree suggests that the photolyase of SpliGV has an evolutionary history different from that of the Plusiinae-infecting group II NPVs**.**

Based on these results, we propose a classification of *phr* genes in baculoviruses as has been in use for instance for inhibitor of apoptosis genes (*iap*) in baculoviruses (Luque et al., 2001) or *Cry* genes in *Bacillus thuringiensis (*http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt). The baculovirus *phr* classification is based upon the degree of relatedness and the phylogenetic analysis (Table 2, Fig. 3A). The group containing ChchNPV-NL *phr1*-like genes is named *phrA*; the one with ChchNPV-NL and ChchNPV-SP2 *phr2*-like genes is named *phrB*; the one with ThorNPV-B9 and PsinNPV-GT2 *phr* genes is named *phrC*.and the SpliGV *phr* gene is so far the sole member of the *phrD* group.

To verify the evolutionary status of the Plusiinae-infecting NPVs that were obtained from various sources, the phylogeny of their polyhedrin sequences was constructed to confirm that the analyzed plusiine baculoviruses also formed a single clade in group II NPVs (Fig. 3B). In our polyhedrin analysis as well as in previous phylogenetic studies of baculoviruses (Herniou and Jehle, 2007; Jehle et al., 2006b), the ThorNPV A28-1 isolate was identified in group I NPVs, in which no photolyase genes have been found so far (van Oers and Vlak, 2007). This probably explains why we cannot detect a photolyase gene in this virus. The ThorNPV-B9 isolate, however, belongs to group II NPV based on the polyhedrin sequence. Therefore, we conclude that the ThorNPV A28-1 and B9 isolates are most likely two distinct baculovirus species present in the *T. orichalcea* host.

Two flanking partial photolyase ORFs were recently reported in GenBank for *Clanis bilineata* (Clbi) NPV (Zhu et al., 2009), one representing an N terminal domain, the other a C terminal domain of PHR. We did not include these incomplete ClbiNPV PHR ORFs in our analysis, since individually they showed too little overlap with our PCR products to give reliable results. In the polyhedrin tree, ClbiNPV is outside the Plusiinae-infecting group II NPVs as expected (Fig. 3B), and close to *Helicoverpa armigera* (Hear) NPV-G4. ClbiNPV would be the first group II NPV outside the *Plusinae*-infecting viruses with *phr* homologues.

It is known that baculoviruses are in general very vulnerable to UV irradiation and quickly inactivated in the environment (Vail et al., 1999). UV protectants are important ingredients of baculovirus preparations for insect control in the field. Moreover, inactivation rates of baculoviruses correlate well with the dose of UV irradiation (Sun et al., 2004a); (Jones et al., 1993). The Plusiinae-infecting group II NPVs seem to have developed a DNA repair system to protect themselves from such damage, although the role of baculovirus *phr* genes in this process has not been clarified yet. It has been reported that fowlpox virus encodes a CPD-photolyase, which is able to utilize light to repair damage of its genome, thereby restoring the infectivity of UV-damaged virus and promoting its survival in the environment (Srinivasan et al., 2001; Srinivasan and Tripathy, 2005). Recently, the ability of the ChchNPV encoded photolyases have been tested in a DNA repair deficient *E. coli* strain. The complementation experiment showed that ChchNPV-*phr2* encodes a functional CPD photolyase (van Oers et al., 2008). This information may lead to the development of baculovirus-based biological control agents with a reduced UV sensitivity.

In this paper, we investigated and confirmed the presence of photolyase sequences in the genome of an array of Plusiinae--infecting group II NPVs and determined their evolutionary relationship by phylogenetic analyses. The results suggest that all plusiine group II NPVs may contain a photorepair system based on photolyases. The results obtained in this work also aid in the identification and localization of the complete photolyase ORFs in baculoviral genomes and in the genomes of their hosts, to allow the biological characterization of photolyase enzymes.
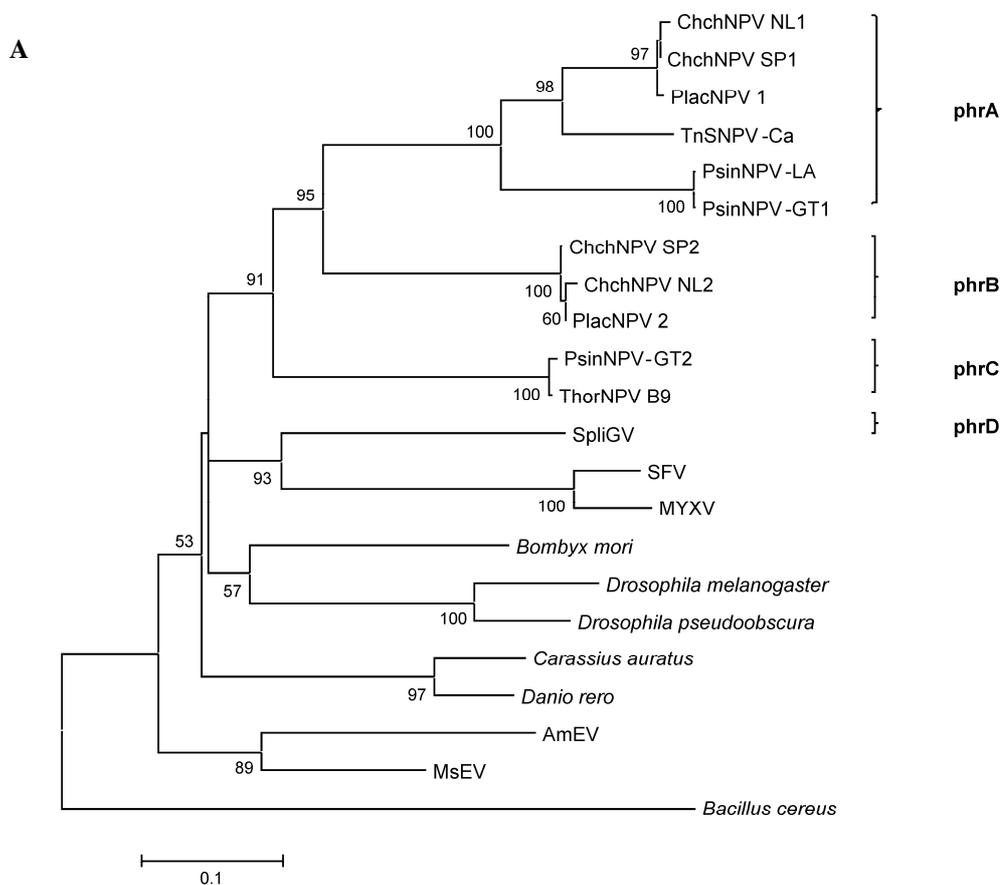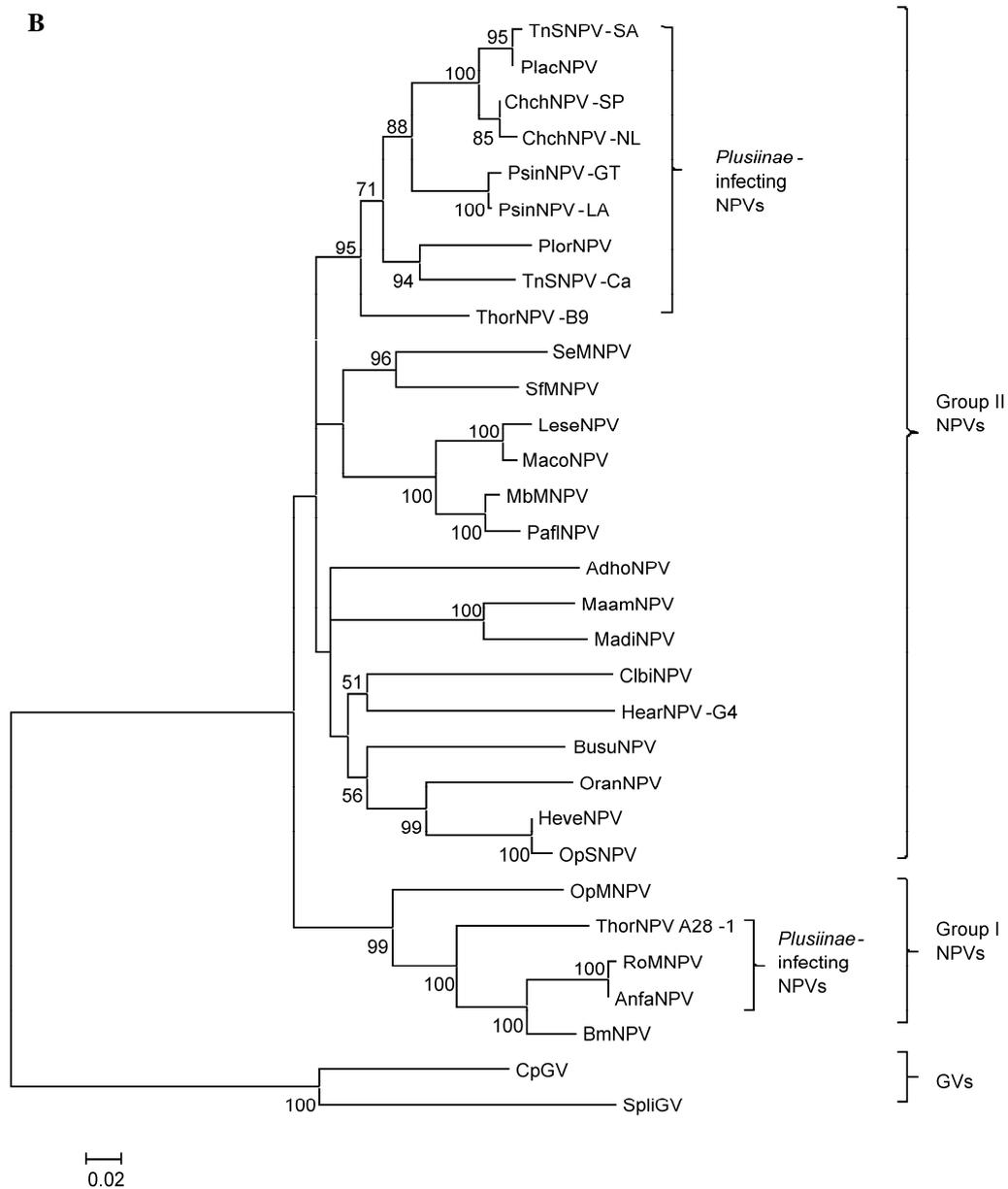
Figure 3. Phylogenetic trees constructed by Neighbor-joining analyses of photolyase and polyhedrin nucleotide sequences. (A) Phylogeny of photolyase; (B) Phylogeny of polyhedrin sequences. Branch support was evaluated by 500 bootstrap replicates. Numbers at the nodes indicate bootstrap scores (scores lower than 50% are not shown). The scales below the topology indicate 0.1 (A) and 0.02 (B) substitutions per site, respectively.

Acknowledgments

Chapter 3

DNA photolyases of *Chrysodeixis chalcites* nucleopolyhedrovirus target to the nucleus and interact with chromosomes and mitotic spindle structures

Abstract

CPD photolyases convert UV-induced cyclobutane pyrimidine dimers in DNA into monomers using visible light as energy source. Two *phr*-genes encoding class II CPD photolyases PHR1 and PHR2 have been identified in *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV). Transient expression assays in insect cells showed that PHR1-EGFP fusion protein was localized in the nucleus. Early after transfection PHR2-EGFP was distributed over cytoplasm and nucleus, but over time it localized predominantly in the nucleus. Immunofluorescence analysis with anti-PHR2 antiserum showed that already early after transfection non-fused PHR2 was mainly present in the nucleus suggesting that the fusion of PHR2 to EGFP hindered its nuclear import. Both PHR-EGFP fusion proteins strongly colocalized with chromosomes and spindle, aster and midbody structures during host cell mitosis. When PHR2-EGFP-transfected cells were superinfected with *Autographa californica* (Ac) MNPV, the protein colocalized with virogenic stroma, the replication factories of baculovirus DNA. The collective data support the supposition that the PHR2 protein plays a role in baculovirus DNA repair.

Keywords: DNA photolyase, *phr*, DNA repair, localization, DNA binding, baculoviruses

1. Introduction

The genome of the baculovirus *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) harbors two genes, Cc-*phr1* and Cc-*phr2*, which encode putative DNA repair enzymes (van Oers et al., 2005; van Oers et al., 2004). Based on sequence comparisons these genes encode DNA photolyases specific for cyclobutane pyrimidine dimers (CPD) and it has been suggested that these genes are involved in viral DNA repair (van Oers et al., 2005). CDPs as well as pyrimidine 6-4 pyrimidone photoproducts ((6-4)PP) are formed upon exposure of genomic DNA to ultraviolet light (UVB, 280-320 nm), but CPDs constitute the majority of UV-induced DNA lesions (Mitchell and Nairn, 1989; Rycyna and Alderfer, 1985; Setlow and Carrier, 1966). Dimeric pyrimidine photoproducts are cytotoxic as they block transcription, may induce mutations and trigger apoptosis (Chigança et al., 2002; Ljungman and Zhang, 1996; Nishigaki et al., 1998).

Pyrimidine dimers can be repaired by several pathways including photoreactivation (see Essen and Klar (2006) for a review), nucleotide excision repair (NER) (reviewed by Schul et al., 2002) and base excision repair (reviewed by Kimura et al., 2004). Photoreactivation is the simplest and fastest DNA repair pathway, which uses only visible light as energy source and involves a single enzyme, either a CPD or a (6-4)PP photolyase. Photoreactivation occurs in all groups of organisms, except placental mammals, which have to rely on the NER pathway to repair pyrimidine dimers (Yasui et al., 1994).

CPD photolyases are monomeric proteins of 450-550 amino acids and carry a flavin adenine dinucleotide (FAD) molecule as catalytic co-factor. Besides FAD, many CPD photolyases also contain a light-harvesting co-factor, which is either 5,10-methenyltetrahydrofolate (MTHF) or an 8-hydroxy-7,8-didemethyl-5-deazaflavin (8-HDF). The light-harvesting co-factor captures near-UV or blue light photons. The excitation energy is transferred to FAD and used to donate an electron to a CPD, leading to the conversion of the pyrimidine dimer into monomers (reviewed by Deisenhofer, 2000; Essen and Klar, 2006; Hearst, 1995; Sancar, 1994; Sancar, 2003).

CPD photolyases have traditionally been categorized into two classes, class I and II, based on the comparison of their amino acid sequences. Phylogenetic analysis suggests that both type I and type II CPD photolyases have a common ancestor but diverged early in evolution (Yasui et al., 1994). The two CPD photolyases encoded by the baculovirus ChchNPV share 45% identity on the amino acid level and belong to class II (van Oers et al., 2005; Xu et al., 2008). Homologues of the ChchNPV *phr* genes have been found in *Trichoplusia ni* SNPV (Willis et al., 2005) and *Spodoptera litura* granulovirus (SpliGV) (Wang et al., 2008) ChchNPV and TnSNPV are closely related viruses, which infect insects of the Plusiinae subfamily of the Noctuidae. Recent studies revealed that *phr* genes are conserved in plusiine-infecting group II NPVs (Xu et al., 2008) of the genus *Alphabaculovirus* (Jehle et al., 2006a).

The activity of the ChchNPV PHR proteins has been examined in complementation assays in a DNA repair deficient *E. coli* strain, showing that *Cc-phr2* encodes an active CPD photolyase (van Oers et al., 2008). In contrast, the photolyase deficiency in *E. coli* could not be rescued with *Cc-phr1*. Cc-PHR2 photolyase also repaired CPD dimers *in vitro* and spectral measurements demonstrated the presence of FAD. Reconstitution experiments suggested that Cc-PHR2 uses a 8-HDF as second co-factor (van Oers et al., 2008). To be able to repair DNA damage in a baculovirus genome, PHR proteins need to be targeted to the nucleus, as baculoviruses replicate and assemble in the nucleus of insect cells, and they should have the ability to bind DNA.

In the current study, the intercellular localization of the two ChchNPV PHR proteins was studied using enhanced GFP fusion constructs transiently expressed in insect cells. The intracellular localization of the CC-PHR-EGFP proteins was determined based on the distribution of the EGFP fluorescence signal. Moreover, the effect of baculovirus infection on the localization of the CC-PHRs was investigated, as the presence of viral proteins and large amounts of viral DNA during infection might influence the targeting of these photolyases. The cellular distribution of Cc-PHR2 was also studied by immunofluorescence to support the observations with EGFP.
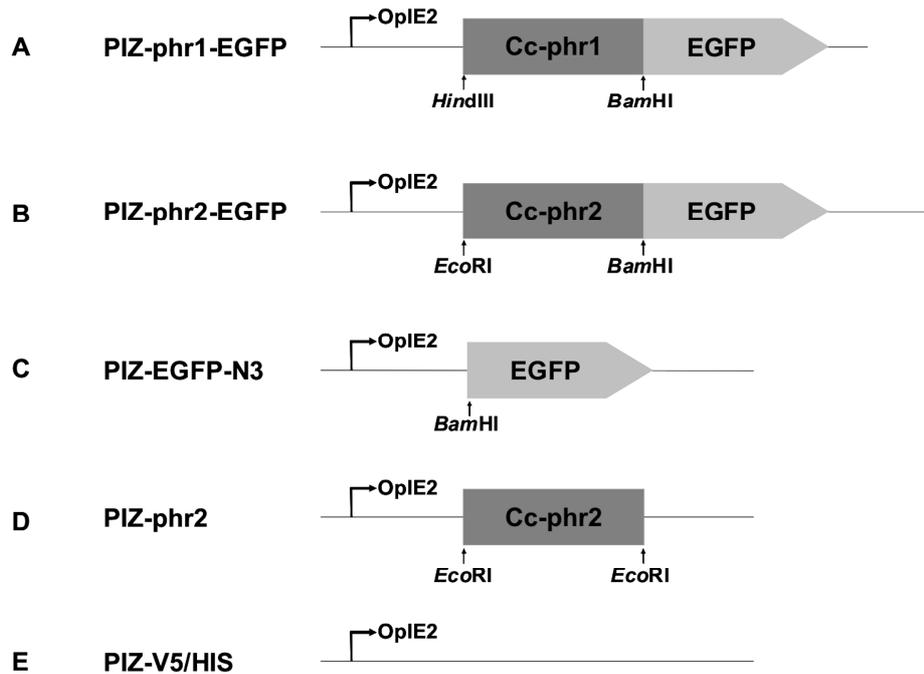
Figure 1. Schematic diagrams of the various constructs used in the study. The ChchNPV *phr1* (A) and *phr2* (B) ORFs were fused to the N-terminus of EGFP under control of the OpMNPV OpIE2 promoter. The non-fused EGFP (C) served as control. The *phr2* ORF was cloned into PIZ-V5-HIS vector for immunofluorescence assays (D), for which the vector (E) served as control.

2. Materials and Methods

2.1. Plasmids

The coding region of Cc-*phr1* was amplified by PCR with Phusion high-fidelity DNA polymerase (Finnzymes) using genomic DNA of the Dutch ChchNPV isolate (ChchNPV-NL) as template. A *Hin*dIII restriction site was introduced upstream of the open reading frame (ORF) and the stop codon was replaced with a *Bam*HI restriction site (see Supplementary Table for primer sequences). The amplicons were cloned into pGEM-T (Promega) and the sequence was verified. The *phr1* fragment was subcloned between the *Hin*dIII and *Bam*HI sites located downstream of the *Orgyia pseudotsugata* (Op) MNPV immediate early 2 (*ie2*) promoter and upstream of the EGFP reporter gene in the vector PIZ-EGFP-N3 to generate PIZ-phr1-EGFP (Fig. 1A). In a similar way, the coding region of the Cc-*phr2* gene flanked by *Eco*RI and *Bam*HI restriction sites was amplified and cloned into PIZ-EGFP-N3 between the corresponding restriction sites to give PIZ-phr2-EGFP (Fig. 1B). The pIZ-EGFP-N3

vector has been made by Dr M. Westenberg by cloning the EGFP sequence of pEGFP-N3 (Clontech) as a *Hin*dIII-*Not*I fragment into the corresponding sites of PIZ-V5/His (Invitrogen). PIZ-EGFP-N3 was also used as control (Fig. 1C). The complete open reading frame of Cc-*phr2* with stop codon and flanked by *Eco*RI sites was recloned from a *phr2*-containing pGEMT-easy vector (van Oers et al., 2008) into the *Eco*RI site of PIZ/HIS-V5 (Invitrogen) resulting in PIZ-phr2 (Fig. 1D). For clarity, none of these coding regions was fused to the His-tag or the V5 epitope as originally present in the PIZ-V5/HIS plasmid (Fig. 1E).

2.2. Anti Cc-PHR2 polyclonal antiserum

In order to produce rabbit polyclonal antiserum against Cc-PHR2, the corresponding *phr2* gene was overexpressed in a photolyase deficient *E. coli* strain (KY29) (Akasaka and Yamamoto, 1991) used with kind permission of Dr. Kazuo Yamamoto. The overexpression and purification methods have been described before (van Oers et al., 2008). Purified Cc-PHR2 (490 μg in 1 ml PBS) was emulsified with 1 ml Freund's incomplete adjuvant (Sigma) and used to immunize two rabbits. These rabbits had been selected for low signal in the pre-immune serum on Western blots using lysates of ChchNPV-infected *T. ni* High Five cells and human cell extract. After 3 booster injections with the same amount of antigen, sera were collected and stored at -80 ℃ after adding 0.1% sodium azide.

2.3. Cell transfection and infection

Trichoplusia ni High Five cells (Invitrogen) were maintained as monolayer cultures in Express Five serum-free medium (Invitrogen) supplemented with 10% L-glutamine (Gentaur). For transfections, 10^5 *T. ni* High Five cells were seeded in 35 mm Petri dishes (Nunc) in Express Five serum-free medium with 10% L-glutamine. After attachment of the cells, the medium was replaced with Grace's non-supplemented insect medium (Sigma), to which 10% L-glutamine was added. After incubation at 27 ℃ for 24 h, cells were transfected with 4 μg plasmid DNA using 10 μl cellfectin reagent (Invitrogen). After incubation at 27 ℃ for 3 h, the transfection mixture was replaced with 2 ml Express Five serum-free medium with 10% L-glutamine, penicillin (30 μg/ml) and streptomycin (75 μg/ml). At 24 h post transfection (p.t.) the cells were infected with *Autographa californica* (Ac) MNPV (E2 strain; Smith and Summers, 1978) at a multiplicity of infection (MOI) of 10 TCID$_{50}$ units/cell. Cells were further incubated at 27 ℃ until further analysis.

2.4. Western blot analysis

Transfected cells were harvested at 24 h p.t. and the proteins were separated by 10% SDS-PAGE and transferred to immobilon-P membrane (Millipore) by semi-dry electrophoretic transfer. The membranes were blocked overnight in 5% non-fat milk in PBS buffer with 0.1% Tween 20. The membranes were incubated with rabbit anti-Cc-PHR2 antibodies diluted 1:500 in PBS with 0.5% non-fat milk and 0.1% Tween 20 for 1 h at room temperature. After three washes with the same buffer, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Dako, Denmark) diluted 1:2000 was used as secondary antibody for 1 h. After washing, blots were stained with nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP) (Roche) in AP buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM MgCl$_2$).

2.5. Confocal laser scan microscopy

EGFP fluorescence in transfected cells and cells that were subsequently infected, was examined with a Zeiss LSM510 confocal laser scanning microscope using a FITC filter. For immunofluorescence cells

were collected at 24 h p.t. and allowed to attach to 0.05% poly-L-lysine coated microscope slides. The cells were fixed in 96% ethanol for 20 min at room temperature and washed with PBS. After blocking for 30 min with PBS containing 10% bovine serum albumin, the cells were incubated with rabbit anti-Cc-PHR2 antiserum, 1:500 diluted in PBS with 1% BSA for 1 h at room temperature. Cells were washed three times with PBS. Rhodamine-linked goat-anti-rabbit IgG secondary antibodies (Molecular Probes) (diluted 1:200, 1 h at 37 ℃ in the dark) were used to detect the primary antibodies. After washing, imaging was performed with a Zeiss LSM510 confocal laser scanning microscope using a Rhodamine filter.

Figure 2. Subcellular localization of the PHR1-EGFP fusion protein in transiently transfected *T. ni* High Five cells. (A-D) Time course fluorescence micrographs of transiently expressed PHR1-EGFP. (F-I) Time course fluorescence examination of PHR1-EGFP, following an AcMNPV infection at 24 h post transfection (p.t.). (K-N) Overlay images of fluorescence (F-I) and phase contrast micrographs. The localization of non-fused EGFP under the various conditions is shown on the right (E, J, O)

3. Results

3.1. Localization of PHR1 in insect cells

To investigate the subcellular localization of ChchNPV photolyases in insect cells, expression plasmids were constructed to produce the PHR proteins fused to the N-terminus of EGFP (Fig. 1A, B). A non-fused EGFP construct served as control (Fig. 1C). After transfection into insect cells the PHR1-EGFP fusion was predominantly localized in the nucleus from 28 h until 120 h p.t. (Fig. 2A-D). Non-

fused EGFP, on the other hand, was homogeneously distributed over the cytoplasm and the nucleus at all time points analyzed as shown for 96 h p.t. (Fig. 2E).

To determine if baculovirus infection affected the subcellular localization of PHR1, cells were infected with AcMNPV at 24 h p.t. and examined for EGFP fluorescence during the course of infection. In the early phase of AcMNPV infection (4 h p.i.) (Fig. 2F, K), the PHR1-EGFP fusion protein showed a similar nuclear localization as at 28 h p.t. without infection. At 24 h p.i. when virogenic stroma appeared in the center of the nucleus of infected cells (Fig. 2G, L), the fluorescence was uniformly present in the cytoplasm as well as in the nucleus. At 48 and 72 h p.i. many viral occlusion bodies or polyhedra were seen in the nucleus of infected cells and fluorescence was seen in both the cytoplasm and nucleus (Fig. 2H-I, 2M-N). Strong fluorescence was not observed in virogenic stroma or in the viral occlusion bodies. The distribution of non-fused EGFP was not affected by AcMNPV infection until polyhedra were produced, when a reduced nuclear EGFP fluorescence was observed (Fig. 2J, O).

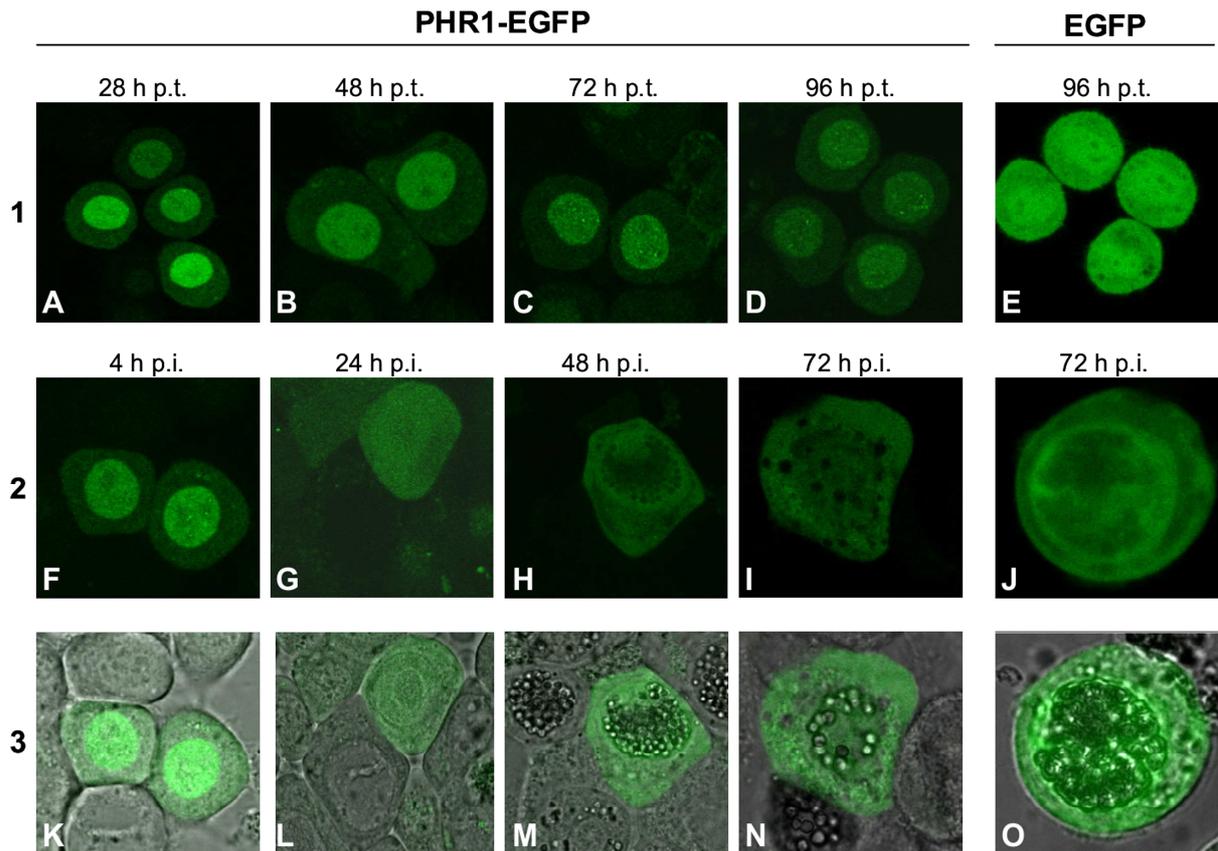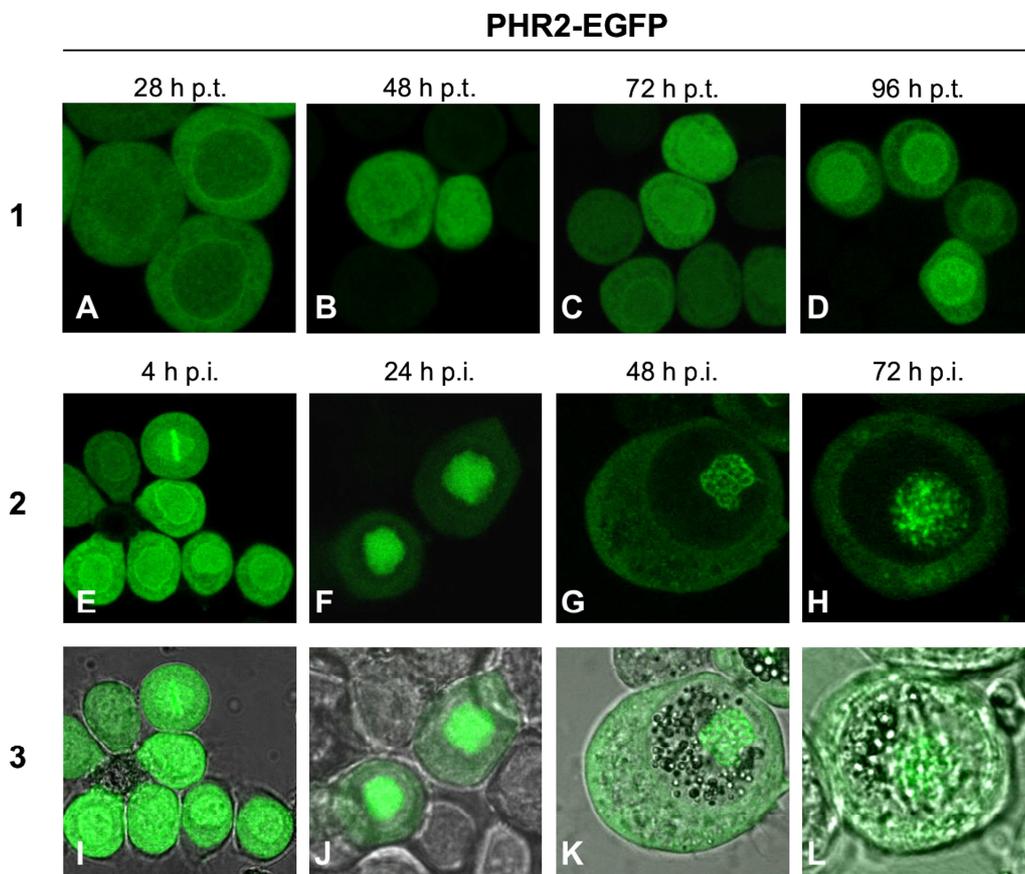**Figure 3.** Subcellular localization of PHR2-EGFP fusion proteins in transiently transfected *T. ni* High Five cells. (A-D) Time course fluorescence micrographs of transiently expressed PHR2-EGFP. (E-H) Time course fluorescence detection of PHR2-EGFP with AcMNPV infection at 24 h p.t. (I-L) Overlay of fluorescence (E-H) and phase contrast micrographs. For non-fused EGFP see Fig. 2.

3.2. Localization of PHR2 in insect cells

In a similar way cells transfected with PIZ-phr2-EGFP were examined by confocal laser scanning microscopy at various time points after transfection (Fig. 3). Initially, the PHR2-EGFP fusion protein

was found in the cytoplasm as well as in the nucleus (Fig. 3A, B). In the cytoplasm an intense ring-shaped fluorescence was seen along the nuclear membrane, especially at 28 h p.t. (Fig. 3A). Over time, the PHR2-EGFP protein accumulated in the nucleus. At 72 and 96 h p.t., the nucleus was marked by intense fluorescence, although some fluorescence still remained in the cytoplasm (Fig. 3C, D). When cells were infected with AcMNPV following transfection, a substantial amount of PHR2-EGFP was detected in the nucleus of infected cells at 4 h p.i. (28 h p.t.) (Fig. 3E). At 24 h p.i., 48 and 96 h p.i., some PHR2-EGFP fusion protein was still detected in the cytoplasm but the majority localized in a central area of the nucleus, colocalizing with virogenic stroma (Fig. 3F-H) in contrast to PHR1. Viral occlusion bodies or polyhedra did not show green fluorescence (Fig. 3K, L).

3.3. ChchNPV photolyases during mitosis

In the transient expression assays both PHR1 and PHR2-EGFP fusion proteins associated clearly with chromosomes of *T. ni* High Five cells during mitosis. Complete mitotic cycles could be visualized by tracing the PHR2-EGFP protein in the cell (Fig. 4), as chromosome binding by the PHR2-EGFP fusion protein was readily evident in the prophase (Fig. 4A), prometaphase (Fig. 4B), metaphase (Fig. 4C) and anaphase (Fig. 4D). During cell division, the mitotic spindle as well as the aster structure were also visible by green fluorescence (Fig. 4C, D), but with much less intensity than the chromosomes. New nuclear membranes were indicated by ring-shaped fluorescence appearing at the telophase (Fig. 4F). The midbody, the remainder of the spindle, was also marked by green fluorescence (Fig. 4E, F). Chromosome binding by PHR1-EGFP was also observed during mitosis but with relatively weak intensity compared to PHR2-EGFP, which may be explained by lower expression levels of *phr1-egfp* in the cells. Less PHR1-EGFP accumulation was observed in all confocal studies and was confirmed by Western blot analysis with anti-EGFP antiserum (data not shown).
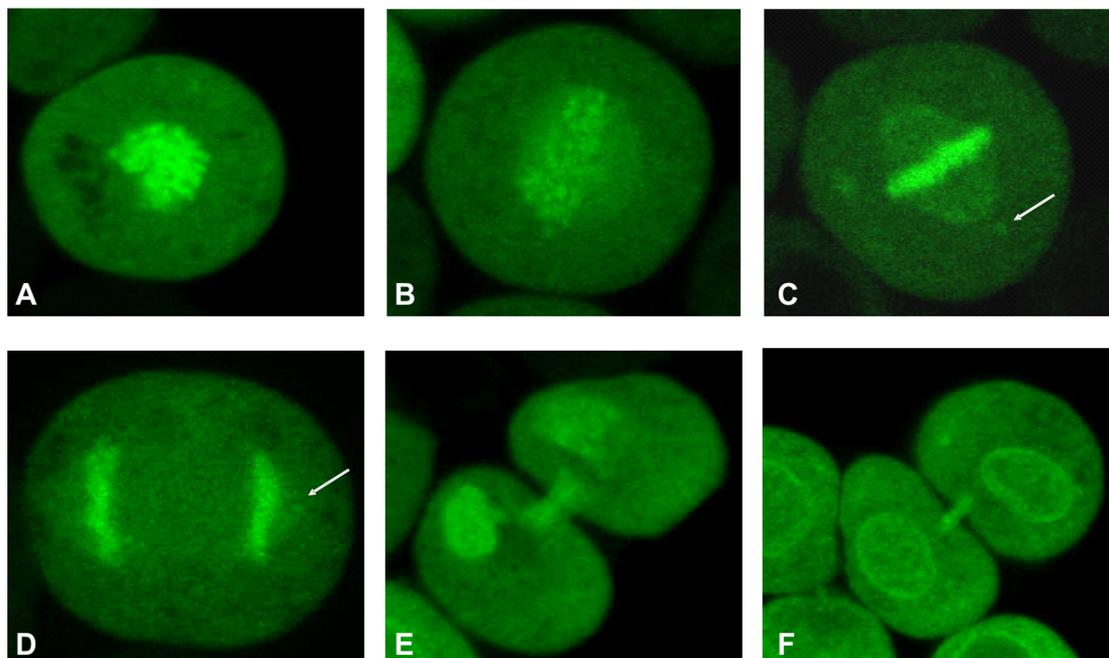

Figure 4. Chromosome binding of PHR2. PHR2-EGFP expressing cells were analyzed at 24 h p.t. PHR2-EGFP is associated with chromosomes, spindle and aster structures during the various stages of mitosis (A-F). Aster structures are indicated by arrow heads. Remains of the polar spindles can be seen as midbody (E, F) towards the end of cell division.

3.4. Location of PHR protein in transfected cells using photolyase specific antibodies

Expression of *phr2-EGFP* and non-fused *phr2* in transfected *T. ni* High Five cells was analyzed by Western blot analysis at 24 h p.t. using polyclonal PHR2 antiserum. This serum was raised by immunization of rabbits with purified recombinant PHR2 protein. The analysis of total extracts of PIZ-phr2 (Fig. 1D) transfected-cells revealed that the antibody recognized non-fused PHR2 as a 53 kDa protein, slightly different from the predicted value of 57.3 kDa, but with similar electrophoretic properties as recombinant PHR2 produced in bacteria (Fig. 5, lanes 1 and 2). The PHR2-EGFP fusion protein was detected as a single ~83 kDa protein (Fig. 5, lane 3), also slightly smaller than the predicted value of 85 kDa. These results verified that the anti-PHR2 antibody recognizes PHR-2 in cell extracts. It also shows that PHR2-EGFP was present as a full length fusion protein. Western blot analysis performed with anti-eGFP antibody confirmed the expression of full length fusion products for both PHR1-EGFP and PHR2-EGFP (data not shown). The anti-PHR2 antiserum showed a weak cross-reactivity to a ~55 kDa cellular protein as judged from its appearance in cells transfected with the empty vector, PIZ-V5/HIS (Fig. 5, lane 4).
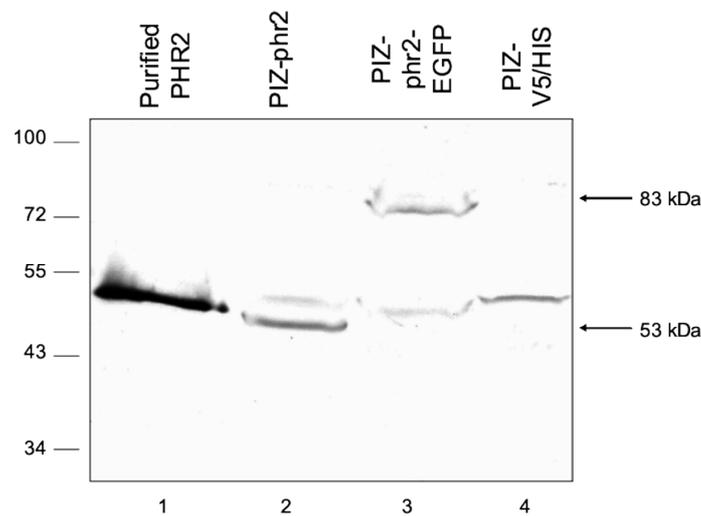
Figure 5. Immunoblot analysis of PHR2 and PHR2-EGFP expression in transfected *T. ni* High Five cells using anti-Cc-PHR2 antibody. Purified recombinant PHR2 was used as control (lane 1). *T. ni* High Five cells were transfected with PIZ-phr2 (lane 2), PIZ-phr2-EGFP (lane 3), or PIZ-V5/HIS (lane 4). A size marker is indicated on the left in kDa. The observed molecular mass of PHR2 (53 kDa) and the PHR2-EGFP fusion protein (83 kDa) is indicated on the right.

3.5. Nuclear localization of PHR2 by immunofluorescence analysis

After testing the performance of the anti-PHR2 antibody by Western blot analysis of transfected cells, the localization of non-fused PHR2 in transfected cells was studied by immunofluorescence analysis. Rhodamine fluorescence revealed that the transiently expressed non-fused PHR2 protein was primarily localized in the nucleus and already present there at 24 h p.t. (Fig. 6A). At this time point the PHR2-EGFP fusion protein was detected with the antiserum in both the cytoplasm and the nucleus with a homogenous distribution (Fig. 6D). The PHR2-EGFP localization was similar as described above in the EGFP-fluorescence studies (see also Fig. 3) as can be seen in a merge image of green and red fluorescence (Fig. 6E, F). Mock-transfected cells and cells transfected with PIZ-V5/HIS only showed a very weak Rhodamine fluorescence, which should be regarded as background labeling (Fig. 6B, C).
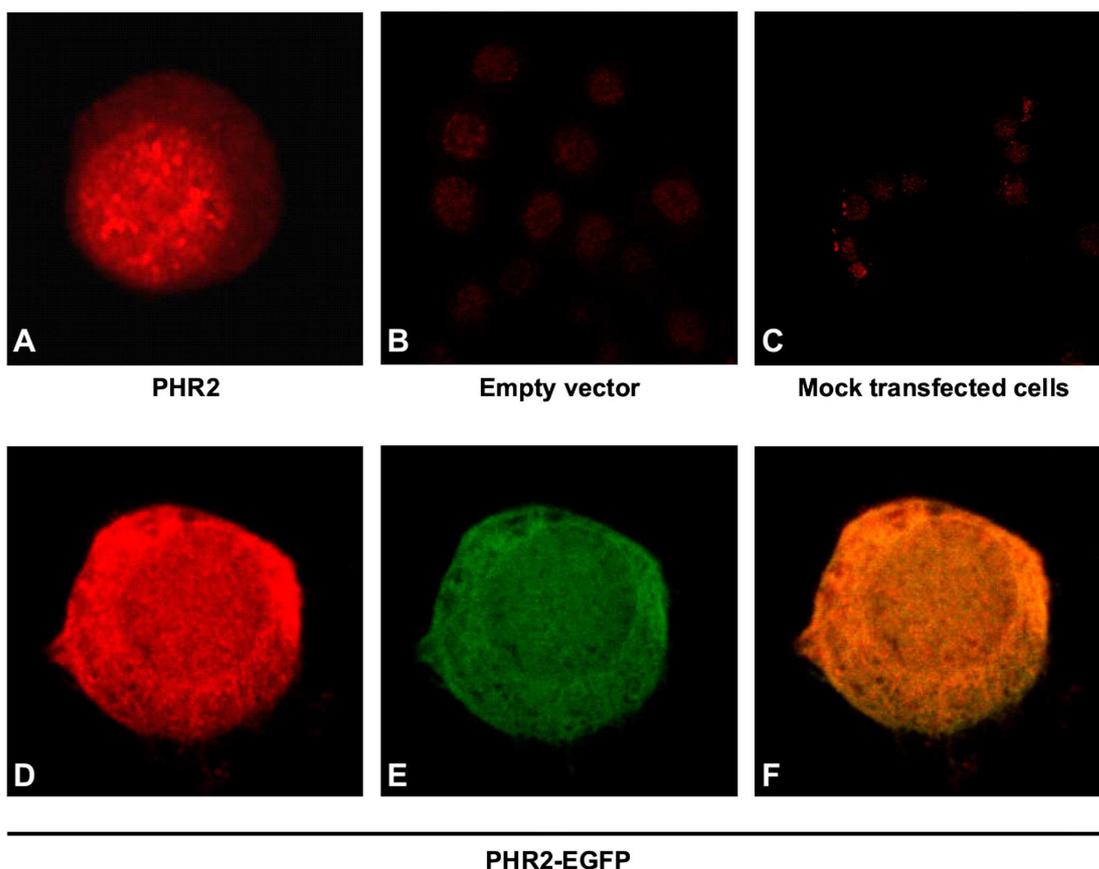
Figure 6. Subcellular localization of PHR2 and PHR2-EGFP in transected *T. ni* High Five cells by immunofluorescence using anti-Cc-PHR2 serum. Rhodamine fluorescence signals the expression of PHR2 at 24 h p.t. (A), while no signal is observed in cells transfected with PIZ-V5/HIS (B) and mock transfected cells (C). Rhodamine fluorescence of cells expressing PHR2-EGFP at 24 h p.t. (D) is compared to EGFP fluorescence (E), and both images have been merged (F). Panels (B) and (C) were taken at low magnification to show that all cells lack fluorescence.

4. Discussion

Baculoviruses are used widely as biocontrol agents of insect pest. However, these viruses are very sensitive to and quickly inactivated by UV irradiation (Fritsch and Huber, 1985; Ignoffo and Garcia, 1992; Vail et al., 1999). The inactivation rate of baculoviruses correlates well with the UV dose received (Sun et al., 2004a). A functional CPD photolyase may repair pyrimidine dimers and thereby increase the UV resistance of progeny virions and enhance their persistence in the environment. Therefore, an active photolyase would be an ecological benefit for a baculovirus and ChchNPV seems to encode two such enzymes.

In this paper, the subcellular localization of Cc-PHR1 and Cc-PHR2 was examined in *T. ni* High Five cells using a transient expression system with EGFP as a reporter. PHR1-EGFP was predominantly localized in the nucleus and the distribution pattern did not change over time. Compared to PHR1-EGFP, it took longer for PHR2-EGFP fusion protein to localize completely in the nucleus (Figs. 2 and 3). The expression level of *phr2-egfp* was much higher than that of *phr1-egfp*, as judged by the difference in intensity of the EGFP fluorescence in the cells and confirmed by Western

blot analysis. This may explain why not all PHR2-EGFP protein was imported in a short period of time into the nucleus. When cells were infected with virus, nuclear import was accelerated leading to the hypothesis PHR2-EGFP may require viral chaperones to enter the nucleus. However, when non-fused PHR2 was analyzed by applying immunoflurescence methods, it was transported efficiently into the nucleus (Fig. 6) showing that the fusion of PHR2 to EGFP is responsible for the distribution of the fusion product over nucleus and cytoplasm.

In this study, the effect of virus infection on the localization of Cc-PHR proteins was analyzed to simulate the more natural situation of virus infection. The idea behind this is that other viral proteins or the presence of large amounts of replicating viral DNA may affect the intracellular distribution of the PHR proteins. The choice was made to infect the transfected cells with the baculovirus AcMNPV, since a fully permissive cell line for ChchNPV is not available. Trials with ChchNPV infections in the semi-permissive *T. ni* High Five cells showed, despite the limitations, that PHR-EGFP fusion proteins were also routed to the nucleus in these cells (unpublished data).

In the presence of a virus infection the PHR1-EGFP fusion product was found in the nucleus and in the cytoplasm with an approximately homogeneous distribution (Fig. 2). PHR2-EGFP primarily localized to a central region of the nucleus in the late phase of AcMNPV infection, which most likely corresponds to the virogenic stroma. This is considered as a *de novo* product of baculovirus infection in which viral DNA is replicated and progeny virions are assembled (Williams and Faulkner, 1997). The enhanced nuclear import and association of PHR2 with virogenic stroma might be explained by the potential interaction between PHR2 and certain viral proteins or the capability of PHR2 to bind to newly synthesizing or synthesized viral DNA. In a previous study, we used oligonucleotides with thymine-dimers and showed that the PHR2 protein was able to repair these dimers, which does imply a direct interaction with DNA (van Oers et al., 2008). No fluorescence was found in polyhedra upon infection with AcMNPV (Fig. 3), which may indicate that PHR2 (as well as PHR1) is not incorporated in progeny virions, or at least not in large amounts. This is in line with the fact that we were unable to show the presence of PHR2 or PHR2-EGFP in polyhedra isolated from these cells by Western blotting (not shown). However, low levels of PHR proteins may not be detectable in this way and the fluorescent data may be affected by absorption of light from the laser beam or the emitted fluorescence by the polyhedrin protein surrounding the virus particles.

PHR1 showed a different intranuclear distribution in infected cells than PHR2. A previous study showed that PHR1 was not an active photolyase when tested in a bacterial system deficient in DNA repair (van Oers et al., 2008). This may be explained by the fact that a conserved tryptophan, which may be involved in electron transfer (Aubert et al., 2000), is absent in PHR1 (van Oers et al., 2008). Whether PHR1 is an active photolyase in insect cells or baculovirus particles is not known, but a preference for virogenic stroma, as seen for PHR2, was not found in our study. Despite these differences, PHR1 colocalized with chromosomes in non-infected dividing cells in a similar manner as PHR2, suggesting that both PHR proteins are capable of binding to condensed DNA.

ChchNPV PHR proteins also colocalized with mitotic spindle structures and midbodies, although the intensity of this fluorescence was low compared to the fluorescence of the chromosomes. From the primary function of DNA photolyases, namely repairing DNA lesions, interaction with DNA is crucial, but interactions with spindle structures consisting of microtubules can not be explained up to now. It is known though that many proteins bind to the spindles, among these are also nucleic acid binding proteins (Sauer et al., 2005).

In this paper, we showed the nuclear localization of the two CPD photolyases encoded by ChchNPV in a transient insect cell expression system. The observed nuclear localization and DNA

binding is a prerequisite for a protein with a proposed function in the repair of baculovirus DNA. This is also the first report in which the interaction of CPD photolyases with chromosomes and spindle structures during mitosis is visualized. The interaction of the viral PHRs with chromosomes and with virogenic stroma for PHR2 suggests that they can bind to condensed DNA, a situation that exists in baculovirus nucleocapsids, both in incoming parental virions and in assembling progeny nucleocapsids (Rohrmann, 2008a). The next goal is to investigate whether or not baculovirus photolyases end up in baculovirus virions.

Acknowledgments

Chapter 4

Protein composition of the occlusion derived virus
of *Chrysodeixis chalcites* nucleopolyhedrovirus

Abstract

Chrysodeixis chalcites nucleopolyhedrovirus (ChchNPV) is a group II NPV and its genome has 151 predicted open reading frames. In this study, the protein composition of ChchNPV occlusion derived virus (ODV) was determined by LC-MS/MS. Fifty-three proteins were identified in ChchNPV ODVs. One ODV-protein is encoded by a gene so far unique to ChchNPV (*Chch105*). The two cyclobutane pyrimidine dimer (CPD)-specific DNA photolyases PHR1 and PHR2, which are characteristic for ChchNPV and thought to be involved in DNA repair, were not detected in the ODVs. A comparison of the ODV proteins identified in ChchNPV and in the ODV proteomes of two other alpha- and one deltabaculovirus showed ten conserved ODV proteins (ODV-E18, ODV-E56, ODV-EC27, ODV-EC43, P6.9, P33, P49, P74, GP41, and VP39). In addition, the *per os* infectivity factors PIF1, PIF2 and PIF3 were present too and can be considered as conserved ODV proteins as well. The sensitive LC-MS/MS method detected twenty-two viral proteins, which have not been identified as ODV proteins in previous studies, despite the fact that homologues are encoded in other baculovirus genomes.

Keywords: baculoviruses, ChchNPV, proteomics, LC-MS/MS, DNA photolyase

1. Introduction

The *Baculoviridae* form a large family of rod-shaped viruses, which infect arthropods (Miller, 1997b). Baculoviruses have a large double-stranded, circular DNA genome and replicate in the nucleus. Over the years, more than 600 baculoviruses have been reported from a variety of species from the insect orders Lepidoptera, Hymenoptera and Diptera. The family *Baculoviridae* is taxonomically divided into four genera (Jehle et al., 2006a). The genera *Alphabaculovirus* and *Betabaculovirus* harbor the lepidopteran nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs), respectively. Baculoviruses that infect insects in the order Hymenoptera and Diptera are classified in the genera *Gammabaculovirus* and *Deltabaculovirus*, respectively. The lepidopteran NPVs are subdivided into group I and group II NPVs based on phylogeny (reviewed by Herniou and Jehle, 2007) and the nature of the budded virus (BV) envelope fusion protein (reviewed by van Oers and Vlak, 2007).

During the infection cycle two morphotypes of virions are produced, BVs and occlusion derived virus (ODV). ODVs are released from occlusion bodies (OB) in the alkaline gut and initiate primary infections in midgut epithelial cells of susceptible insects, while BVs spread the virus infection from cell to cell leading to systemic infection within an infected host insect (reviewed by Miller, 1997b; van Oers and Vlak, 2007). Knowledge of the protein composition and assembly of these virus particles is crucial to understand the biology of the virus and the role that individual proteins play during infection.

Mass spectrometry-based proteomics can be used as a tool to identify and study structural and functional viral proteomes (Maxwell and Frappier, 2007). It is a powerful tool, because it is very sensitive, can deal with complex protein mixtures and offers analysis with high throughput (Aebersold and Mann, 2003; Domon and Aebersold, 2006). In recent years, two mass spectrometry approaches, matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry and liquid chromatography-linked tandem mass spectrometry (LC-MS/MS), have been used to analyze the protein composition of purified virions (Maxwell and Frappier, 2007).

As of February 2010, genome sequences of fifty-two baculoviruses have been reported (based on the NCBI database). The availability of these data facilitates proteomic analysis of baculoviruses by providing open reading frame (ORF) databases. The determination of the protein composition of ODVs from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV, group I NPV) revealed forty-four ODV proteins (Braunagel et al., 2003). Forty-four ODV-associated proteins were also detected in *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV), a dipteran NPV (Perera et al., 2007). A recent paper on the ODV proteome of the group II NPV *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) showed twenty-three structural proteins (Deng et al., 2007), a remarkably lower number. All these studies were performed with MALDI-TOF MS analysis. This raises the question what are the conserved viral proteins present in the ODV, which contribute to the structure and scaffold of ODV and what is the role of the unique proteins in the ODV.

In the current study, we used LC-MS/MS analysis to determine the protein composition of purified ODVs of the baculovirus *Chrysodeixis chalcites* (Chch) NPV. The genome of ChchNPV has a size of 149,622 bp and has 151 predicted ORFs (van Oers et al., 2005), which include eight so far unique baculovirus genes. Most noteworthy of this virus is the presence of two ORFs (*Cc-phr1* and *Cc-phr2*) encoding putative class II cyclobutane pyrimidine dimer (CPD) DNA photolyases (van Oers et al., 2005; van Oers et al., 2004). The activity of the ChchNPV PHR proteins has been examined in complementation assays in a DNA repair deficient *E. coli* strain, showing that *Cc-phr2* encodes an active CPD photolyase (van Oers et al., 2008).

If the PHR proteins are actively engaged in viral DNA repair, it can be postulated that they function before or upon entry, but at least prior to viral DNA replication. In that case they should be either present in the ODV or be expressed early after infection. The LC-MS/MS approach provides a sensitive method to determine whether Cc-PHR proteins are present in ODVs. To this aim, purified ODV proteins were separated by SDS-PAGE and analyzed by LC-MS/MS techniques. The identified proteins were compared with the results obtained in the proteomics analysis of ODVs of the other three NPVs mentioned above.

2. Methods and materials

2.1. Virus purification

For this research we used the ChchNPV-NL strain, which has been isolated from *Chrysodeixis chalcites* larvae in greenhouses of Applied Plant Research, Naaldwijk, The Netherlands as described before (van Oers et al., 2004). Fourth instar *C. chalcites* larvae were used to propagate the ChchNPV virus. Infected larvae were macerated with distilled water in a glass homogenizer and filtered through cheese cloth. The occlusion bodies (OBs) were washed three times with distilled water. Additional cellular debris was removed by centrifugation over a 25% sucrose cushion, after which the OBs were washed twice with water to remove the sucrose.

To a suspension of 4 x 10^9 OBs in distilled water half a volume of 3 x DAS (0.3 M NA$_2$CO$_3$, 0.5 M NaCl, 0.03 M EDTA, pH 7.5) was added and the OBs were incubated for 10 min at 37 °C. Tris-HCl (pH7.5) was added to this mixture to a final concentration of 0.05 M to neutralize the pH. ODVs were purified over a 25-65% continuous sucrose gradient in 10 mM Tris-HCl, pH 7.5, by centrifugation at 100,000 x *g* (Beckman SW32) for 90 min at 4 °C. The collected ODVs were washed in 0.1 x TE (10 mM Tris/HCl and 1 mM EDTA, pH 7.5) by centrifugation at 40,000 x *g* (Beckman SW32) at 4 °C for 1 h. The pellet was resuspended in 100 µl 0.1 x TE. A small sample was negatively stained with 2% phosphotungstic acid (PTA), pH 6.5, and examined by transmission electron microscopy to confirm the purification of the virions (not shown).

2.2. Protein separation and in-gel digestion

Proteins from purified ChchNPV ODV were separated by 12% one-dimensional SDS-PAGE and stained with colloidal blue (Invitrogen). The gel lane was divided into eight pieces containing proteins with a molecular mass ranging from lower than 10 kDa, 10-26 kDa, 26-34 kDa, 34-43 kDa, 43-55 kDa, 55-72 kDa, 72-130 kDa, to higher than 130 kDa, respectively. The gel pieces were dehydrated with 100% acetonitrile (ACN) and vacuum dried. They were further incubated in 10 mM dithiothreitol in 50 mM ammonium bicarbonate (ABC buffer) at 57 °C for 1 h and in 55 mM iodoacetamide (Sigma) in the same buffer at room temperature also for 1 h. After washing in ABC buffer, in-gel protein digestions were performed with sequencing grade modified porcine trypsin (Promega, Madison, WI) at 37 °C for 15 h, after which the digests were shortly centrifuged at 6000 x *g*. The supernatants were collected and the remaining gel material was extracted with 5% Triflouroacetic acid (TFA), followed by an extraction with 15% ACN / 1% TFA. The extracts were combined with the original supernatants. The materials were vacuum dried and dissolved in 20 µl 0.1 % formic acid.

2.3. LC-MS/MS

The peptides resulting from the trypsin digestion were subjected to LC-MS/MS analysis. To this aim, the samples were concentrated over a 0.10 * 32 mm Prontosil 300-5-C18H (Bischoff, Germany) pre-concentration column at a flow of 6 µl/min for 5 min. An electrospray potential of 3.5 kV was applied directly after the pre-concentration column. The peptides were eluted onto a 0.10 * 200 mm Prontosil 300-3-C18H analytical column with a gradient of 10 to 35% ACN in 0.1 % formic acid at a flow of 0.5 µl/min for 50 min. Full scan positive mode Fourier transform mass spectra (FTMS) were measured between mass-to-charge ratios of 380 and 1400 with a LTQ-Orbitrap spectrometer (Thermo electron, San Jose, CA, USA). MS/MS scans of the four most abundant doubly and triply charged peaks in the FTMS scan were recorded in a data dependent mode in the linear trap.

2.4. Protein identification

The spectra lists obtained were searched against a ChchNPV ORF database with Biowork 3.3.1 (Thermo Fisher Scientific, Inc.). One differential modification per peptide was allowed for oxidation of methionines and de-amidation of asparagine or glutamine residues, while carboxy-amidomethylation of cysteines was set as a fixed modification. Trypsin digestion was set at fully enzymatic with a maximum of 3 missed cleavages allowed. The mass tolerance for peptide precursor ions was set to 10 ppm and for MS/MS fragment ions to 0.5 Da. The theoretical protein database of ChchNPV was used to identify the peptides (NC 007151, created on June 29, 2005, downloaded from www.ncbi.nlm.nih.gov/sites/entrez) as well as a list of commonly observed contaminants. A decoy database was created with the program SequenceReverser from the MaxQuant package (Cox and Mann, 2008). The peptide identifications obtained were filtered in Bioworks with the following filter criteria: ΔCn > 0.8, Xcorr > 1.5 for charge state 2+, Xcorr > 3.3 for charge state 3+ and Xcorr > 3.5 for charge state 4+ (Peng et al., 2003). Only those proteins that showed a Bioworks Score factor (Sf) larger then 0.8 were taken into account.

2.5. Homology search

Comparisons of the identified ChchNPV virion proteins with the three other baculoviruses for which the ODV composition has been studied previously by proteomics (AcMNPV, HearNPV and CuniNPV) (Braunagel et al., 2003; Deng et al., 2007; Perera et al., 2007) were carried out using BLASTP against the NCBI databases of AcMNPV (L22858); HearNPV (AF271059) and CuniNPV (AF403738), respectively.

3. Results and discussion

3.1. ChchNPV ODV proteins

In the current study, LC-MS/MS was employed to determine the protein composition of the ODV particles of ChchNPV. The purified ODVs were separated by SDS-PAGE, divided in mass-based fractions and digested by trypsin. The resulting peptides were analyzed by LC-MS/MS. Reliable matches were obtained by searching against the theoretical ChchNPV ORF database. Peptides derived from the highly abundant polyhedrin protein, which forms the matrix of OBs (Rohrmann 1986), and the polyhedrin envelope protein (PEP) (Chch121) (Gombart et al., 1989; van Lent et al., 1990) were not considered as real components of the ODV proteome. Peptides derived from polyhedrin have also been identified when analyzing the ODV proteome of AcMNPV and HearNPV (Braunagel et al., 2003; Deng et al., 2007).

The details of the ChchNPV ODV proteins identified in this study are summarized in Table 1 in terms of protein coverage, predicted molecular mass, number of matched peptides and predicted function. The coverage of each theoretical protein sequence with a reliable match was in the range of 2.1 to 95.2 %. Although five proteins (LEF-6, V-ubiquitin, FP/25K, Chch46 and Chch47) were identified with only one matched peptide, their presence in ODV is estimated as real due to the stringent filter criteria settings and the reliability of the MS/MS spectra. In total, 53 out of 151 predicted ChchNPV proteins were identified as ODV virion proteins, covering 35.1% of the complete theoretical proteome of ChchNPV (Fig. 1).

Both Cc-PHR1 and Cc-PHR2 proteins were not detected in ODVs in this analysis despite the high sensitivity of the methods. This implies that these proteins are not present in OBs and cannot be active in the virus particle or in the very early stages of the infection. UV-damage is a major constraint in the use of baculoviruses in the field to control insect pests. OBs have to survive outside of their hosts for a variable amount of time and are then sensitive to UV irradiation. UV-protectants are essential components of commercial baculovirus products. If the PHRs are involved in blue light-dependent DNA repair of viral DNA, they should be active early to repair the incoming DNA of ODVs. The observation that they are not find in ODVs, suggest that they are expressed as early genes, in line with the presence of baculovirus early (CAGT; *phr1*) and GATA (phr2) putative promoter motifs (van Oers et al., 2004; 2005).
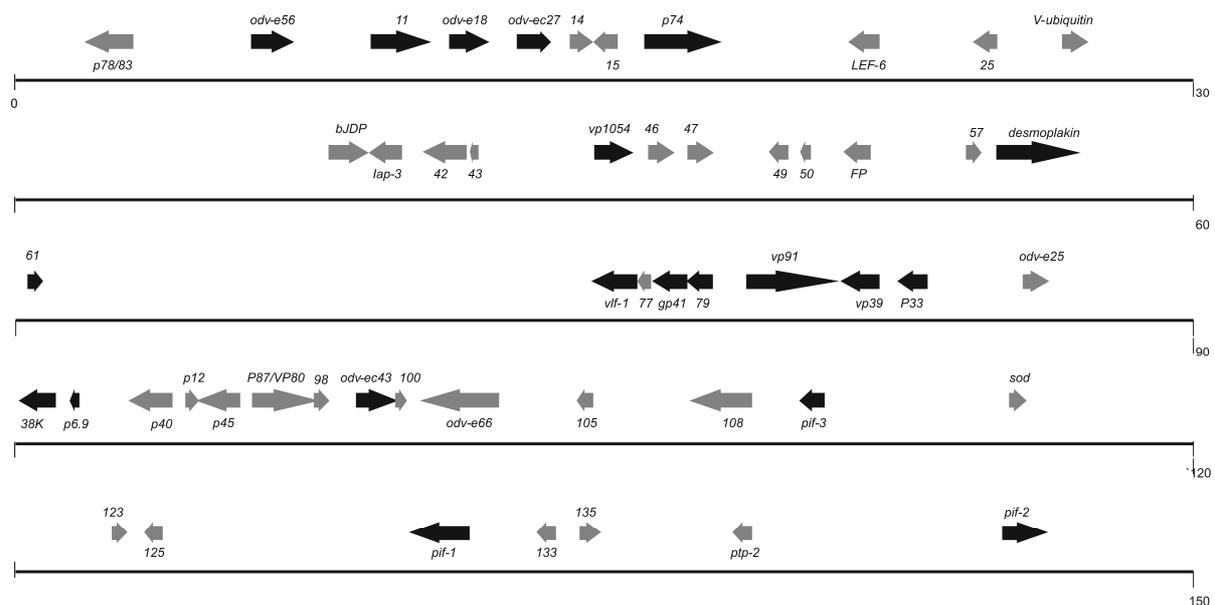
Figure 1. Linearized representation of ODV structural protein ORFs of ChchNPV determined by LC-MS/MS, arrows in grey indicate the positions and the directions of the transcription of proteins. The ORFs conserved in all baculovirus genome are illustrated in black. The number 105 represents a unique gene. Numbers refer to the nucleotide position relative to the start codon of polyhedrin.

A total of twenty identified ChchNPV ODV proteins is encoded by baculovirus core genes, conserved in all sequenced baculoviruses (McCarthy and Theilmann, 2008; van Oers and Vlak, 2007). These are ODV-E18, ODV-E56, P49, ODV-EC27, VP1054, Desmoplakin, Chch61, VLF-1, GP41, Chch79, VP91, VP39, P33, 38K, P6.9, ODV-EC43, PIF1, PIF2, PIF3, and P74. Many of these proteins are known to be involved in virion packaging or assembly, or are crucial for oral infection

(reviewed by Cohen et al., 2009) and show, in the case of the HearNPV homologues, binary interactions using yeast-two-hybrid systems and immunoprecipitation (Peng et al., 2010b).

Proteins identified in the ChchNPV ODV, which have been reported before to be associated with nucleocapsids of both ODV and BV (mostly in AcMNPV), include ODV-EC27 (Vanarsdall et al., 2007), P78/83 (Pham et al., 1993), FP/25K (Braunagel et al., 1999), VP39 (Pearson et al., 1988), 38K (Wu et al., 2006), P6.9 (Wilson and Price, 1988), P40 (Braunagel et al., 2001), P49 (Braunagel et al., 2003), VP80 (Lu and Carstens, 1992; Muller et al., 1990), VLF-1 (Vanarsdall et al., 2006) and VP1054 (Olszewski and Miller, 1997a). The *per os* infectivity factors, essential for oral infection and associated with the ODV-envelope, PIF1 (Kikhno et al., 2002), PIF2 (Braunagel et al., 2003; Pijlman et al., 2003a), PIF3 (Slack and Arif, 2006) and P74 (Faulkner et al., 1997) were all identified. The analysis also revealed the ODV envelope proteins ODV-E18 (Braunagel et al., 1996b; McCarthy and Theilmann, 2008), ODV-EC43 (Braunagel et al., 2003; Fang et al., 2003), ODV-E25 (Russell and Rohrmann, 1993), ODV-E56 (Braunagel et al., 1996a), and ODV-E66 (Russell et al., 1997).

GP41 has been reported to be located in the tegument of the ODV particle and is also an essential protein for infectious BV production (Olszewski and Miller, 1997b). VP91 is found in the ODVs and was reported before as associated with capsid and envelop of ODVs (Russell and Rohrmann, 1997). Desmoplakin, involved in pre-occluded virion and OB formation has been found in ODV nucleocapsids by immunodetection in HearNPV (Ke et al., 2008). PTP2 encodes a putative protein tyrosine/serine phosphatase and homologues were found in for instance *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) and *Mamestra configurata* nucleopolyhedrovirus (MacoNPV-A) (IJkel et al., 1999; Li et al., 2002). This protein putatively removes phosphates from both tyrosine and serine/threonine residues and may regulate the phosphorylation status of a variety of proteins. PTP2 and *Bombyx mori* nucleopolyhedrovirus (BmNPV) PTP share a low identity at the amino acid level (18%). Whether PTP2 has a similar function as the BmNPV PTP in enhancing locomotion of lepidopteran hosts (Kamita et al., 2005) is not known.

Superoxide dismutase (SOD) (Tomalski et al., 1991) was also found in ChchNPV ODV and its predicted function is to remove active oxygen radicals. IAP-3 (Bideshi et al., 1999), an inhibitor of apoptosis is present in the ODV as well. Moreover, Chch105, which is encoded by a unique gene with unkown function, was identified in ODV (Table 1). No information is available for the potential functions of twenty-one other ChchNPV ODV-associated proteins.

In addition to the identified viral proteins, host proteins may be present in the ODV. Since a complete sequenced genome of the host is not available at this moment in time, it is difficult to indentify specific host proteins in the virus particle. However, the detection of peptides that are not related to ChchNPV ORFs, may reflect the potential presence of host proteins. Typical BV-specific proteins such as the envelope fusion protein F (*Chch150*) were not detected, showing that the ODV preparation was not contaminated with BV particles.

3.2. Comparison of the protein composition of baculovirus ODVs

The collection of viral proteins identified in ODV of ChchNPV was compared with the ODV proteome of three other baculoviruses (Table 2). Sixteen proteins (P78/83, ODV-E56, P49, ODV-E18, ODV-EC27, P74, desmoplakin, GP41, VP39, P33, ODV-E25, P6.9, P40, VP80, ODV-EC43 and ODV-E66) are present in the ODVs of AcMNPV, HearNPV and ChchNPV. Essential proteins for viral DNA replication, for instance DNA polymerase (Kool et al., 1994b) and helicase (Kool et al., 1994a; Kool et al., 1995) both of which were identified in the ODVs of AcMNPV and HearNPV (Braunagel et al., 2003; Deng et al., 2007), were not identified in ChchNPV ODVs.

Nineteen ORFs of ChchNPV identified in the current study, have homologues in the genome of the more distantly related CuniNPV (genus *Deltabaculovirus*) (Moser et al., 2001) and ten of these shared proteins (ODV-E56, ODV-E18, P49, ODV-EC27, P74, GP41, VP39, P33, P6.9 and ODV-EC43) have been detected in proteomic analysis of ODVs in all four baculoviruses. All ten are encoded by baculovirus core genes (Table 2). In addition PIF1, PIF2 and PIF3 need to be included as conserved ODV proteins since they are absolutely essential for the midgut infection by ODVs (see reviewSlack and Arif, 2006). Although not all three PIFs were identified by proteomic analysis in ODVs of AcMNPV and HearNPV, either due to their low abundance probably in combination with technical reasons (low sensitivity), they have been recognized as ODV envelop proteins in other studies (Braunagel et al., 2003; Kikhno et al., 2002; Ohkawa et al., 2005; Peng et al., 2010a; Song et al., 2008). We therefore propose to address these thirteen proteins (including PIF1, PIF2 and PIF3) as the core components of baculovirus ODV particles. Four genes that encode ChchNPV ODV proteins (Chch105, Chch123, PTP2 and Chch135) do not have homologues in the genomes of AcMNPV, HearNPV and CuniNPV.

4. Conclusion

In the present study, we identified fifty-three proteins, which are associated with ChchNPV ODV by LC-MS/MS based proteomics. The comparison of the ODV proteomes of four baculoviruses (three alpha- and one deltabaculovirus) revealed that they share thirteen ODV proteins. These proteins are encoded by core baculovirus genes, so genes conserved in all baculoviruses, which suggests that these ODV core proteins perform crucial functions in ODV assembly and/or the biological activity of the ODV particle. A total of twenty ChchNPV ODV proteins have homologues with other baculovirus core genes, whereas four are unique to ChchNPV. The two putative PHR proteins were not identified in the ODV proteome of ChchNPV and their role in the infection process remains enigmatic.

Table 1

Identified ODV proteins of ChchNPV

ORF	Protein name	NCBI accession No.	Number of amino acids	Protein Coverage %	Molecular Mass (kDa)	Number of matched peptide	Function
2	P78/83	YP_249606	417	80.1	46.5	147	Inducing nuclear actin polymerization
7	ODV_E56	YP_249611	358	61.73	39.2	299	ODV envelope protein
11	P49	YP_249615	475	74.9	55.9	438	Structural glycoprotein /Required for BV production
12	ODV-E18	YP_249616	81	12.3	8.6	8	ODV envelope protein
13	ODV-EC27	YP_249617	294	95.2	34.4	171	ODV envelope protein
14	Chch14	YP_249618	93	16.1	11.0	4	Involved in oral infection
15	Chch15	YP_249619	207	25.1	23.5	9	
17	P74	YP_249621	658	37.1	75.4	82	*Per os* infectivity factor, binds to midgut epithelium
21*	LEF-6	YP_249625	157	7.6	18.4	1	Late expression factor 6
25	Chch25	YP_249629	213	12.7	24.8	3	
26*	V-ubiquitin	YP_249630	78	11.5	8.9	1	Signalling protein degradation
38	bJDP	YP_249642	346	63.6	39.9	116	
39	IAP-3	YP_249643	278	12.9	32.3	4	Inhibitor of apoptosis
42	Chch42	YP_249646	373	83.4	42.8	308	
43	Chch43	YP_249647	77	63.6	9.3	11	
45	VP1054	YP_249649	336	19.6	39.6	17	Essential for nucleocapsid assembly
46*	Chch46	YP_249650	85	11.8	9.7	1	
47*	Chch47	YP_249651	102	7.8	11.5	1	
49	Chch49	YP_249653	169	48.5	19.8	88	
50	Chch50	YP_249654	91	65.9	10.6	19	
51*	FP/25K	YP_249655	231	6.9	26.8	1	Involved in ODV yield
57	Chch57	YP_249661	129	25.6	15.6	2	
59	Desmoplakin	YP_249663	717	73.8	81.3	509	Involved in pre-occluded virion and OB formation
61	Chch61	YP_249665	132	41.7	15.1	18	
76	VLF-1	YP_249680	392	44.6	45.9	52	Very late gene expression factor
77	Chch77	YP_249681	122	41.8	13.9	24	

ORF	Protein name	NCBI accession No.	Number of amino acids	Protein Coverage %	Molecular Mass (kDa)	Number of matched peptide	Function
78	GP41	YP_249682	299	86.3	34.1	779	Involved in nucleocapsid formation
79	Chch79	YP_249683	224	7.1	25.9	7	
81	VP91	YP_249685	790	52.8	91.0	131	Viral capsid associated protein
82	VP39	YP_249686	333	83.2	37.6	721	Major capsid protein
84	P33	YP_249688	251	39.0	30.1	23	Putative regulator of apoptosis
86	ODV-E25	YP_249690	225	47.6	25.0	17	ODV envelope protein
91	38K	YP_249695	316	39.2	37.5	31	Required nucleocapsid assembly
93	P6.9	YP_249697	90	36.7	10.5	73	Nucleocapsid core DNA-binding protein
94	P40	YP_249698	380	76.6	42.8	189	Subunit of protein complex
95	P12	YP_249699	117	24.8	13.1	6	Involved in nuclear localization of G-actin
96	P45	YP_249700	378	11.9	44.1	4	
97	P87/VP80	YP_249701	576	73.1	65.3	358	Major capsid protein
98	Chch98	YP_249702	61	14.8	7.3	2	
99	ODV-EC43	YP_249703	356	79.5	41.2	270	Associated with ODV
100	Chch100	YP_249704	95	45.7	11.1	12	
101	ODV-E66	YP_249705	675	61.3	76.3	290	ODV envelope protein, hyaluronan lyase activity
105	Chch105	YP_249709	136	28.7	15.5	6	
108	Chch108	YP_249712	531	55.6	61.0	142	Nucleocapsid- and ODV envelope-associated protein
110	PIF-3	YP_249714	216	31.5	24.1	9	*Per os* infectivity factor 3
115	SOD	YP_249719	151	26.5	16.0	11	Removes active oxygen radicals
123	Chch123	YP_249727	132	56.1	16.0	20	
125	Chch125	YP_249729	438	2.1	51.7	2	
131	PIF-1	YP_249735	519	37.8	58.9	69	*Per os* infectivity factor 1
133	GP16	YP_249737	95	7.4	11.1	6	
135	Chch135	YP_249739	137	7.3	15.6	2	
140	PTP2	YP_249744	164	43.9	18.9	19	Ser/Thr and Tyr protein phosphatases
148	PIF-2	YP_249752	382	47.1	43.6	65	*Per os* infectivity factor 2

*ORF is identified with single matched peptide.

Table 2.

Homologues of ChchNPV ODV proteins in the three other baculoviruses*

ChchNPV		ORF numbers			
ORF	Protein name	AcMNPV	HearNPV	CuniNPV	Reference
2	P78/83	**9**	**2**	-	(Pham et al., 1993)
7 [a, b]	ODV-E56	148	15	102	(Braunagel et al., 1996a)
11 [a, b]	P49	142	9	30	(Braunagel et al., 2003)
12 [a, b]	ODV-E18	143	10	31[c]	(Braunagel et al., 1996b; McCarthy and Theilmann, 2008)
13 [a, b]	ODV-EC27	144	11	32	(Braunagel et al., 1996b; Vanarsdall et al., 2007)
14	Chch14	145	12	-	(Lapointe et al., 2004)
15	Chch15	146	13	-	
17 [a, b]	P74	138	20	74	(Faulkner et al., 1997)
21	LEF-6	28	24	-	(Passarelli and Miller, 1994)
25	Chch25	34	27	-	
26	V-ubiquitin	35	28	-	(Miller, 1997a)
38	bJDP	51	39	-	
39	IAP-3	-	103	-	(Bideshi et al., 1999)
42	Chch42	-	**44**	-	(Deng et al., 2007)
43	Chch43	-	45	-	
45 [b]	VP1054	**54**	47	**8**	(Olszewski and Miller, 1997a)
46	Chch46	55	48	-	
47	Chch47	56	49	-	
49	Chch49	**59**	51	-	
50	Chch50	60	52	-	
51	FP/25k	**61**	53	-	(Harrison and Summers, 1995)
57	Chch57	75	69	-	
59 [b]	Desmoplakin	**66**	**66**	-	(Ke et al., 2008)
61[b]	Chch61	68	64	**58**	
76 [b]	VLF-1	**77**	71	**18**	(Vanarsdall et al., 2006)
77	Chch77	78	72	-	
78 [a, b]	GP41	80	73	33	(Olszewski and Miller, 1997b)
79 [b]	Chch79	81	74	**106**	
81[b]	VP91	**83**	76	**35**	(Russell and Rohrmann, 1997)
82 [a, b]	VP39	89	78	24	(Pearson et al., 1988)
84 [a, b]	P33	92	80	14	(Clem, 2007)
86	ODV-E25	**94**	**82**	-	(Russell and Rohrmann, 1993)
91 [b]	38K	98	86	**87**	(Wu et al., 2006)
93 [a, b]	P6.9	100	88	23	(Wilson and Price, 1988)
94	P40	**101**	**89**	-	(Braunagel et al., 2001)
95	P12	**102**	90	-	
96	P45	103	91	-	
97	P87/VP80	**104**	**92**	-	(Lu and Carstens, 1992; Muller et al., 1990)
98	Chch98	110	93	-	

ChchNPV		ORF numbers			Reference
ORF	Protein name	AcMNPV	HearNPV	CuniNPV	
99 [a, b]	ODV-EC43	109	94	69	(Braunagel et al., 2003; Fang et al., 2003)
100	Chch100	108	95	-	(Chen et al., 2006)
101	ODV-E66	**46**	**96**	-	(Russell et al., 1997)
105	Chch105	-	-	-	
108	Chch108	-	**100**	-	(Deng et al., 2007)
110 [b]	PIF-3	115	98	**46**	(Slack and Arif, 2006)
115	SOD	31	106	-	(Tomalski et al., 1991)
123	Chch123	-	-	-	
125	Chch125	18	-	-	(Wang et al., 2007)
131 [b]	PIF-1	119	**111**	**29**	(Kikhno et al., 2002)
133	GP16	130	119	-	(Gross et al., 1993)
135	Chch135	-	-	-	
140	PTP2	-	-	-	(IJkel et al., 1999; Kamita et al., 2005)
148 [b]	PIF-2	**22**	132	**38**	(Braunagel et al., 2003)

[*] ORF numbers in bold have been identified in the proteomic analyses of AcMNPV, HearNPV or CuniNPV

[a] ORF represents an ODV associated protein also identified in all other three proteomics analyses

[b] ORF is conserved in all sequenced baculovirus genomes

[c] Based on gene clustering (McCarthy and Theilmann, 2008), but similarity to Chch12 is very low

Chapter 5

A baculovirus photolyase enhances the tolerance of
Helicoverpa armigera **nucleopolyhedrovirus to UV irradiation**

Abstract

The broad application of baculoviruses as biocontrol agents is hindered by their susceptibility to inactivation by sunlight. The main reason for this is probably the induction of cyclobutane pyrimidine dimers (CPDs) in the viral DNA by irradiation with the ultraviolet component of sunlight (UV). CPD photolyases repair UV-induced CPD lesions in DNA with the help of blue light, a process called photoreactivation. A sub-group of the baculoviruses encodes one or more DNA photolyases, which are thought to play a role in rescuing this UV damage. To examine whether the DNA photolyase Cc-PHR2 encoded by *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) has a function in reducing the UV sensitivity of the virus, the corresponding open reading frame was incorporated into *Helicoverpa armigera* single nucleocapsid NPV (HearNPV), lacking a photolyase gene, in a bacmid set-up. Incorporation of the Cc-*phr2* gene in HearNPV resulted in a 3-fold increase in mortality over the *phr2*-null virus, when occlusion bodies (OBs) were treated with blue light after UV irradiation. When OBs were kept in the dark this affect was not seen. This increase was found despite the fact that only a small proportion (0.2%) of the viral genome copies turned out to contain the *Cc-phr2* ORF as a consequence of bacmid genome instability during replication. The obtained data support the supposition that Cc-PHR2 also has a photo-repair activity *in vivo* and that the Cc- *phr2* gene is likely to increase the fitness of the baculovirus as it makes the virus less sensitive to UV light.

Key words: CPD photolyase, DNA repair, UV inactivation, UV resistance, *Helicoverpa armigera* nucleopolyhedrovirus, baculovirus, biological control

1. Introduction

Baculoviruses are large double-stranded DNA viruses of insects with circular genomes in the size range of 80-180 kilo base pairs (reviewed by van Oers and Vlak, 2007). In the infection cycle of most baculoviruses, two morphotypes of virions, budded virus (BV) and occlusion derived virus (ODV) are produced. ODVs initiate primary infections in midgut epithelial cells of insect larvae and are responsible for the spread of infection between host insects when encapsided in so-called viral occlusion bodies (OBs). The BV form causes systemic infection by cell-to-cell transmission within an infected insect (Miller, 1997b; Rohrmann, 2008a). Infection of larvae usually results in the production of large amounts of OBs, which are then spread in the environment.

Baculoviruses are pathogenic to insect species of the orders Lepidoptera, Hymenoptera and Diptera, and control the size of insect populations in nature. Since they have in general, a high level of host specificity they can be used against specific insect pests (Ward et al., 2003). Nowadays, a number of baculoviruses have been developed for the protection of crops, forests, pastures and greenhouse crops and flowers (Moscardi, 1999; Szewczyk et al., 2006). Two of the major drawbacks of enhanced use of baculoviruses are their slow speed of action relative to insecticides and their UV sensitivity. Baculoviruses have been genetically engineered to improve their speed of action, for instance insertion of neurotoxins or deletion of viral gene encoding ecdysteroid UDP-glucosyltransferase (*egt*) (Inceoglu et al., 2006 for review), but the application of the modified viruses has been halted as a consequence of biosafety concerns. Although baculoviruses in OBs are stable under most environmental conditions, their sensitivity to inactivation by ultraviolet (UV) light is the other major factor hampering the further application of baculoviruses as biocontrol agents. Attempts towards solving this problem have mainly focused on the addition of UV protection agents to the virus formulation such as carbon black, gelatin and titanium dioxide (Black et al., 1997; Fritsch and Huber, 1985; Ignoffo and Garcia, 1992). These UV blocking agents can provide protection against solar irradiation, but may interfere with the intrinsic ability of baculoviruses to adhere to plant surfaces. The necessity to add such compounds leads to high production costs of the final products (Black et al., 1997) and is not readily compatible with their use in biological and organic cropping systems. In addition, the restricted host range has limited the commercialization of baculoviruses.

The cotton bollworm (*Helicoverpa armigera*) is one of the most serious pests in global cotton growing areas in Australasia and the Indian continent (Fitt, 1989). *H. armigera* nucleopolyhedrovirus (HearNPV) has been developed as commercial biopesticide and is used to control this pest successfully in China since the 1980s (Zhang et al., 1981). HearNPV has been genetically modified by either deleting the *egt* gene from its genome (Chen et al., 2000b) or by introducing a toxin gene from the scorpion *Androctonus australis* (AaIT-toxin) (Sun et al., 2004b). Those recombinant HearNPVs show an improved efficacy in terms of killing speed compared to the wild-type virus, but the recombinants generated so far demonstrated similar inactivation rates upon solar radiation (Sun et al., 2004a) and improvements are being sought.

Baculovirus DNA repair mechanisms were not known until the discovery of photolyases genes in the baculovirus *Chrysodeixis chalcites* (van Oers et al., 2004). Cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4 PPs) are formed upon exposure of DNA to ultraviolet light (UVB). The CPDs form the majority of the photoproducts (around 70%) formed by UV radiation (Mitchell and Nairn, 1989; Rycyna and Alderfer, 1985; Setlow and Carrier, 1966). Photoreactivation is the most efficient pyrimidine dimer repair system, since it requires a single enzyme, which is either a CPD or a (6-4) photolyase, using only blue light as energy source (reviewed

by Essen and Klar, 2006). CPD photolyases are divided into two classes based on the divergence of their amino acid sequences. Photoreactivation is conserved in all groups of organisms except placental mammals (Yasui et al., 1994), which rely on the nucleotide excision repair pathway to repair DNA damages (reviewed by Schul et al., 2002). CPD photolyases carry a flavin adenine dinucleotide (FAD) as catalytic co-factor. Most CPD photolyases also contain a light-harvesting co-factor, which is either 5,10-methenyltetrahydrofolate (MTHF) or an 8-hydroxy-7,8-didemethyl-5-deazaflavin (8-HDF) (reviewed by Deisenhofer, 2000; Essen and Klar, 2006; Park et al., 1995), which captures the blue light photon.

Two genes, Cc-*phr1* and Cc-*phr2*, which encode putative class II CPD photolyases, were identified in the genome of the baculovirus *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) (van Oers et al., 2005; van Oers et al., 2004). The activity of the ChchNPV PHR proteins has been examined in a DNA repair deficient *E. coli* strain, showing that *Cc-phr2* encodes an active CPD photolyase (van Oers et al., 2008). Cc-PHR2 produced in bacteria can repair CPD dimers *in vitro* as well (van Oers et al., 2008), but the function of these baculovirus photolayses in their natural system remains to be elucidated. One way to investigate this is the deletion of *phr* genes from ChChNPV and to test its sensitivity to UV radiation. However this is complicated since no recombineering system is readily available to make knock-out mutants in this virus. The alternative is to engineer *phr* genes into a baculovirus lacking *phr* genes, such as HearNPV (Chen et al., 2001).

To achieve this goal, the *Cc-phr2* open reading frame (ORF) was brought under the control of the *Orgyia pseudotsugata* (Op) MNPV immediate early 2 (*ie2*) promoter and introduced into the HearNPV genome in a bacmid set-up (Wang et al., 1997). An early promoter was chosen, since the expression of *Cc-phr2* is predicted to be early (van Oers et al., 2004) and OpIE2 is a proven early promoter in a variety of invertebrate cell systems (InsectSelect System with PIZ/V5-His, Invitrogen) The resistance of HearNPV-phr2 to UV radiation was determined in comparison to wild type HearNPV by determining the percent mortality of *H. armigera* larvae fed with UV-irradiated OBs.

2. Methods and materials

2.1. Cells and Insects

 HzAM1 cells were maintained at 27 °C in Grace's insect medium (GIBCO, Invitrogen) supplemented with 10% fetal bovine serum (pH 6.0). The HearNPV bacmid (HaBacHZ8) used in this study was constructed and tested by Dr. Hanzhong Wang (Wang et al., 2003). A laboratory colony of the cotton bollworm, *H. armigera*, was reared on artificial diet at 28 ± 1 ºC at a 16-h light/8-h dark photoperiod (Zhang et al., 1981).

2.2. Construction of recombinant viruses

Starting with the HearNPV bacmid constructed by Wang et al. (2003) the HearNPV *polyhedrin* gene needed to be reinserted together with the *Cc-phr2* gene. A fragment containing the HearNPV polyhedrin (*polh)* coding region, together with its native promoter and poly-A sequence was amplified by Phusion high-fidelity PCR (Finnzymes) using genomic DNA of HearNPV-G4 (Sun et al., 1998) as template with primers Ha-phF and Ha-phR (Table 1). Restriction sites for *Sma*I and *Pst*I were introduced up - and downstream of the *polh* fragment, respectively. The amplicons were cloned into pGEM-T (Promega) and the sequence was verified. The HearNPV *polh* fragment was subcloned between the *Sma*I and *Pst*I sites in pFastBacDual (Invitrogen), thereby replacing the *Autographa*

californica multicapsid nucleopolyhedrovirus (AcMNPV) *polh* promoter with the HearNPV *polh* gene and its promoter, generating pFBD-Ha-polh.

The OpMNPV *ie2* promoter and an *egfp* reporter gene were derived as *Bsp*HI (the end made blunt with T4 polymerase) – *Xho*I fragment from the vector pIZ-EGFP-N3 (Xu et al., 2010) and subcloned into pFBD-Ha-polh between *Sma*I and *Xho*I sites resulting in pFBD-egfp-polh, in which the AcMNPV p10 promoter was replaced with the OpMNPV *ie2* promoter + *egfp*. From this vector pFBD-Ha-polh + *ie2* promoter (in short pFBD-polh) was generated by deleting the *egfp* gene as an *Nco*I fragment. The complete open reading frame of Cc-*phr2* was derived from PIZ-phr2 (Xu et al., 2010) and cloned into pFBD-polh in the *Eco*RI site downstream of the *ie2* promoter to produce pFBD-phr2-polh. The two plasmids, pFBD-polh and pFBD-phr2-polh, were used to make recombinant HaBacHZ8 bacmids by transposition. Recombinant bacmids were identified by PCR with M13 primers, according to the manufacturer manual (Invitrogen). The recombinants were named Hear-polh and Hear-phr2-polh, respectively.

2.3. Transfection and infection

HzAM1 cells were seeded at a density of 5 x 10^5 cells per 35 mm diameter tissue culture dish (Nunc). Cells were transfected with 1 µg recombinant bacmid DNA (Hear-polh or Hear-phr2-polh) according to the Bac-to-Bac Expression System manual (Invitrogen) using 15 µl Lipofectin (Invitrogen). At 6 days post-transfection (p.t.) 1 ml of culture medium from the transfected cells was centrifuged at 950 x *g* for 5 min to remove cell debris. The supernatant was used to infect a 25 cm^2 fresh HzAM1 cell culture (50% confluent) and subsequently scaled up to a 75 cm^2 HzAM1 cell culture. The cells containing OBs were harvested at 10 d post infection (p.i.) by centrifugation at 2000 x *g*. OBs of the recombinant HearNPVs were propagated *in vivo* by feeding 100 newly molted *H. armigera* third instars with infected OB-containing cells. The OBs were purified from dead larvae as previously described (Sun et al., 2003). The concentration of the OB suspension was determined using a hemocytometer (0.05 x 0.05 mm).

2.4. Detection of Cc-phr2 transcripts

To verify the transcription of *Cc-phr2* in the Hear-phr2-polh infected HzAM1 cells, cells were harvested at 6 and 12 h p.i. These time points were chosen on the basis of a transcription time course study of the OpMNPV *ie-2* gene (Theilmann and Stewart, 1993). Total RNA was extracted using Trizol (Invitrogen) and then treated with DNase (Promega). One microgram RNA was used to synthesize first-strand cDNA using an oligo dT anchor primer (Table 1) and Moloney murine virus reverse transcriptase (Promega). Subsequently, standard PCR was performed with the *Cc-phr2* specific primers phr2-RTF and phr2-RTR (Table 1) to amplify a 113-bp fragment. As a negative control for *Cc-phr2* transcription, a similar RT-PCR was also performed on RNA isolated from Hear-polh infected cells. To exclude amplification of viral genomic DNA, a reaction was performed in which the RT step was omitted. The identity of the RT-PCR product was confirmed by automated sequencing (Invitrogen).

2.5. Quality control of viruses amplified in vivo

Total viral DNA was extracted from OB suspensions obtained from larvae and BVs from the 75 cm^2 HzAM1 cell culture mentioned above, respectively. Viral DNA was extracted from 40 µl OB suspension by incubation with 0.1 M Na_2CO_3, 0.01 M EDTA and 0.17 M NaCl for 30 min at 37 ºC. The pH was neutralized with 10 µl 1 M Tris/HCl, pH 7.5. The suspension was boiled for 10 min

followed by centrifugation at 16,000 x *g* and the supernatant containing viral DNA was collected. One volume 20% PEG8000 in 1 M NaCl was added to 500 μl BV-containing medium and the suspension was then centrifuged at 16,000 x *g* for 15 min. BVs in the pellet were dissolved in 20 μl water and then disrupted in 80 μl lysis buffer (10 mM Tris/HCl pH7.6; 10 mM EDTA; 0.25% SDS) and 5 μl protease K at 50 °C for 1 h. DNA of BVs was extracted with phenol/chloroform (1:1) and then ethanol precipitated and dissolved in water. The Hear-polh and Hear-phr2-polh DNA samples derived from OBs and BVs were analyzed by PCR with the primers G4phF and G4phR (Table 1), which anneal upstream (nt position: 131014-131032) of the *polh* promoter and downstream (nt position: 1259-1279) of the poly-A sequence of the *polh* gene in wt HearNPV, respectively. Neither of the primer binding sites is present in the DNA of the recombinant HearNPVs. The genomic DNA of wild-type HearNPV (G4) served as positive control and Hear-phr2-polh bacmid DNA as negative control. The expected amplicon in wild type DNA is 1671 bp in size.

2.6. Quantification of recombinant Hear-phr2-polh by real-time PCR

Quantitative real-time PCR was employed to determine the proportion of genome copies of Hear-phr2-polh in OBs prepared to infect larvae for the UV sensitivity assay. SYBR-Green-I-based qPCR reactions (Qiagen) were performed as described (Zwart et al., 2008b) with an annealing temperature of 55 °C. Specific primers for *Cc-phr2* (phr2-RTF and phr2-R, see Table 1), for the *egt* gene present in the genomes of both wild-type and recombinant HearNPV (egt-F and egt-R, Table 1), and for the mini-F replicon specific for the bacmid insert (see Zwart et al., 2008a) were used. Relative copy numbers of *Cc-phr2, egt* and mini-F were determined by comparative analysis with RotorGene 6.0 software (Corbett Research; Sydney, Australia). Bacmid DNA of Hear-phr2-polh was used to calibrate the number of *phr2* copies. The proportion of genome copies that carry *phr2* in the virus mixture is calculated by the ratio *Cc-phr2* copies: *egt* copies. The fraction recombinant bacmid in this mixture is determined by the ratio mini-F copies: *egt* copies. The corresponding standard error in the ratio was estimated as described before (Zwart et al., 2008b).

Table 1.

Oligonucleotides used in this study

Oligonucleotides	**Sequence from 5'to 3'**
Ha-phF	CTTCCCGGGTATCATGAAGGATTTGATAGATGACGATG
Ha-phR	ATACTGCAGCGTCAGAATCGGAAAACGGGT
Oligo dT anchor primer	AAGCAGTGGTATCAACGCAGAGTAC(T)$_{30}$VN
Phr2-RTF	GAGCAGGCGCTCGAGTATTCT
Phr2-RTR	GCTTCTATCGTGAAGACCGCAA
G4phF	CACGCTGAAGATTGACTGC
G4phR	GATTTTACAACGACGCATAGCCGTTC
phr2-R	GCAAATGCTCCACATGCAACC
egt-F	CCGTTGACTGCATTCCGCCTTG
egt-R	ATTGTGGAAGACCGATGGACTGCC

2.7. Determination of active UV dose

To determine the UV dose needed to inactivate the recombinant HearNPV (Hear-phr2-polh and Hear-polh) OBs, suspensions of 2.5 x 10^8 OBs per ml water were irradiated *in duplo* in 35 mm Petri dishes (Nunc) with UV light (250 nm) at doses of 0, 200, 250, 300, 1230, 1968 and 2460 J/m^2, respectively. The irradiated virus suspension was kept in the dark for 6 h. Subsequently, *H. armigera* 3rd instars (48 insects per treatment) were fed with pieces of artificial diet (1 mm x 1 mm) coated with treated OBs at the same dose for each virus (5 x 10^6 OBs per insect). When no UV light is present, this dose is sufficient to kill all larvae, as the LD99 for the early staged 3rd instars is 6700 OBs/larvae (Sun et al., 2004b). Prior to the infection, larvae were starved in 24-well tissue culture plates for 24 h to secure synchronous and complete ingestion. The larvae were cultured individually at 28 ± 1 ºC and checked daily for mortality.

2.8. Laboratory bioassays for UV resistance

The UV resistance of Hear-phr2-polh and Hear-polh was compared at the lethal dose determined for these viruses of 2460 J/m^2. A treatment at 0 J/m2 served as control for any differences in lethal infective dose between these two viruses. The irradiated samples were either incubated in complete darkness or were illuminated with visible light with a regular 8W TL-tube at 28 ± 1 ºC for 6 h. An 8 mm glass plate was used to filter out short wavelength UV light. The treated OBs were used to infect larvae as described above. The larvae fed OBs treated with light or kept in the dark after UV irradiation, were kept in white light or in darkness, respectively, until the diet coated with OBs was completely consumed. After that they were put on a normal night/day schedule as outlined above. The assay was performed twice independently.

3. Results

3.1. Transcription ability of Cc-phr2 in Hear-phr2-polh inoculated HzAM1 cells

A recombinant HearNPV bacmid was constructed that contained the *Cc-phr2* ORF under the control of an OpMNPV *ie2* promoter immediately upstream but in opposite orientation of the original *polh* locus (Fig. 1a). The native *polh* promoter of HearNPV and the complete *polh* coding region were inserted to allow the generation of OBs (Fig. 1). A *Cc-phr2* null construct served as negative control (Fig. 1b).

Once recombinant viruses were amplified in HzAM1 cells, a RT-PCR study was performed to verify that the gene for DNA photolyase was indeed specifically transcribed. A 113 bp transcription product corresponding to the *Cc-phr2* gene was detected at 6 h p.i. in the RNA isolated from HzAM1 cells infected with Hear-phr2-polh, and much more RT-PCR product of *Cc-phr2* was obtained at 12 h p.i. This product was absent from Hear-polh infected cells. When RNA was used for PCR without an RT step, only a very weak amplification signal was seen, which was probably due to residual viral DNA in the preparation (Fig. 2).
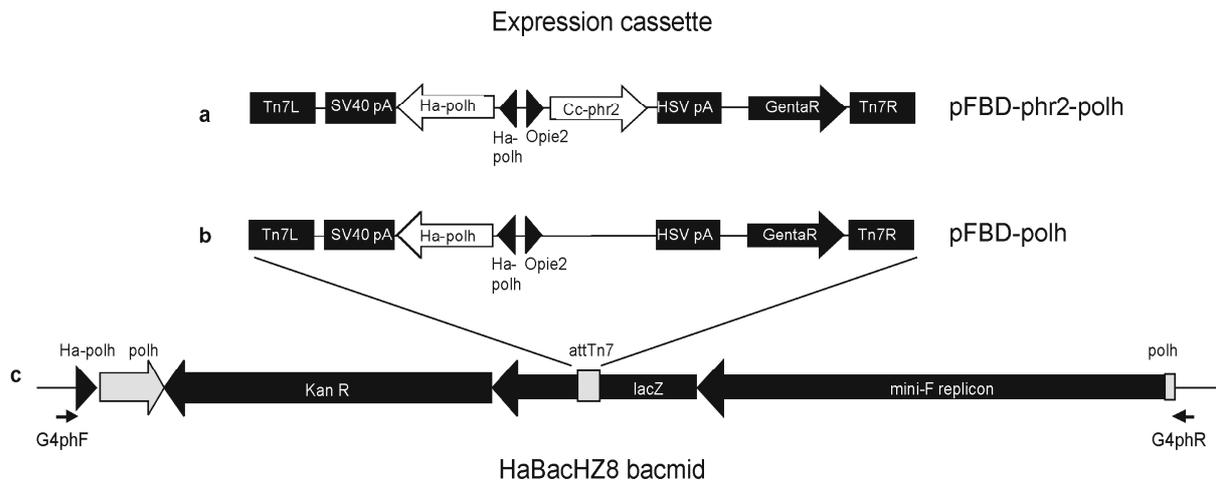
Expression cassette

Figure 1. Construction of recombinant HearNPV bacmids. The expression cassette in the donor plasmid (pFBD) contains the *Cc-phr2* ORF under the control of the OpMNPV ie2 promoter. The promoter, the ORF and the poly-A sequence of the HearNPV *polh* gene are introduced in opposite orientation of *Cc-phr2* (a). The *phr2* null bacmid (b) served as a control. The HaBacHZ8 bacmid vector (c) contains the kanamycin resistance gene (Kan R), the mini-F replicon for maintenance in *E. coli*, and a LacZ cassette with a transposon integration site (attTn7) for bacterial transposon Tn7. The expression cassette in the donor plasmid is transposed into HaBacHZ8 at the attTn7 site to generate a recombinant bacmid.
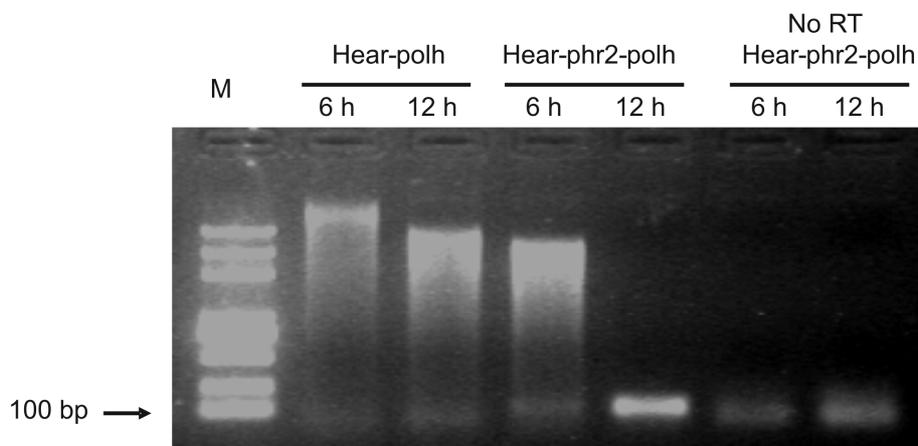

Figure 2. Transcriptional analysis of *Cc-phr2* by RT-PCR. RNA was isolated from HzAM1 cells infected with recombinant Hear-polh or Hear-phr2-polh at 6 h p.i. and 12 h p.i. RT-PCR was performed using the *Cc-phr2* specific primers. The expected PCR product is 113 bp in size. A PCR without RT step was also performed.

3.2. Identification of recombinant HearNPVs

The recombinant viruses were propagated in third instars of *H. armigera* and OBs were purified. Prior to analyzing the UV sensitivity of these viruses in a bioassay, a PCR analysis was performed to determine whether the OBs of the recombinant HearNPVs were pure after propagation in larvae. It is known that insect larvae can contain latent baculoviruses, which can be inactivated upon superinfection (Cooper et al., 2003; Hughes et al., 1993). An approximately 1.7 kbp PCR product was obtained signaling the presence of wild-type HearNPV (G4 isolate) in both the ODVs of Hear-phr2-polh and Hear-polh. To determine at what stage this wild-type virus first occurred in the samples, the

same PCR was performed with BV samples harvested at each passage in cell culture. The results showed that wild-type HearNPV genomes already existed in the BV-samples taken from the culture medium of bacmid-transfected cells, although they were absent in the bacmid DNA used for transfection (Fig. 3). Hence, the PCR results demonstrated that both BVs and OBs of the recombinant viruses were contaminated with wild-type HearNPV. When the ratio of bacmid genomes versus all viral genomes was determined (mini-F to *egt* copies) it was shown that approximately 96% of the viruses in the recombinant HearNPV stock did not contain the bacterial cassette. In addition, *Cc-phr2* had been deleted in around 93% of the remaining mini-F containing recombinant viruses based on the ratio of *phr2* to mini-F copies. Therefore, approximately 0.2% of the viral genome copies in the OB stock contained the *Cc-phr2* gene. This strongly suggests that the wild type HearNPV was already generated during the replication in cell culture.
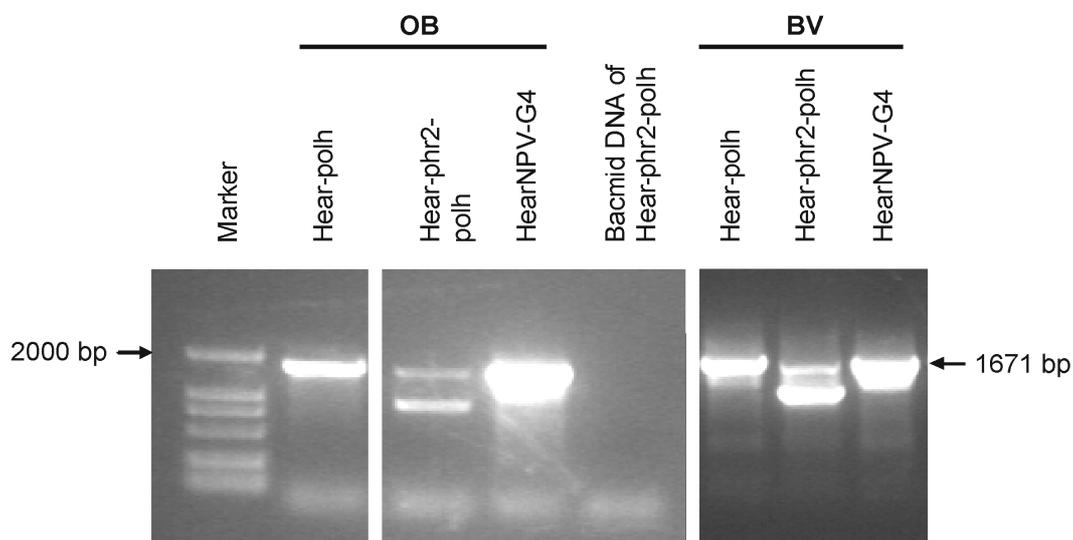

Figure 3. Quality control of recombinant viruses by PCR. Genomic DNA was isolated from OBs and BVs of wild-type and recombinant HearNPVs, respectively. PCR was performed using primers, which are not able to anneal to the DNA sequence of the recombinant viruses. The bacmid DNA of Hear-phr2-polh derived from *E. coli* served as negative control.

3.3. Cc-PHR2 improved the UV resistance of HearNPV

The UV dose needed to inactivate the recombinant viruses was determined based on the percent mortality observed when feeding UV-irradiated OBs to *H. armigera* larvae with a fixed dose of 5 x 10^6 OBs (Table 2), more than enough to kill 100% of the infected larvae. Hear-phr2-polh and Hear-polh OBs both induced 100% mortality upon increasing UV doses in the range of 0 - 1230 J/M^2, indicating that these UV doses were insufficient to inactivate the recombinant OBs. When the viruses received a dose of 1968 J/M^2, the mortality of each virus declined slightly but still stayed at a high level (> 93%). The mortalities of both recombinant viruses decreased strongly with 2460 J/M^2 UV light, which suggested that 2460 J/M^2 of UV irradiation inactivated the majority of recombinant ODV in the OBs (Table 2).

Hence, the assay to determine whether Cc-PHR2 can rescue inactivation by UV light was performed with a dose of 2460 J/M^2. In the assay, the non-irradiated OBs (0 J/M^2) served as control and in that case 100% mortality was obtained for each recombinant, as expected. Mock-infected

control larvae survived and pupated. No significant differences were obtained for Hear-polh kept in the dark or exposed to visible light after UV irradiation with an average mortality of 31.3% and 26.5%, respectively (Table 3). When Hear-phr2-polh was kept in the dark, no significant difference was measured with Hear-polh with 24% average mortality. However, 88% of the larvae on average died of oral inoculation with light-treated Hear-phr2-polh, which was significantly higher than the average values from Hear-polh (dark and light) and Hear-phr2-polh (dark) (Table 3). The assay clearly indicated that the presence of *Cc-phr2* contributed to the rescue of HearNPV from UV inactivation.

Table 2

Mortality (%) of H. armigera larvae when fed with recombinant HearNPV OBs treated with UV light at different doses

Virus	UV dose (J/M^2)						
	0	200	250	300	1230	1968	2460
Hear-polh	100	100	100	100	100	97.9	23.4
Hear-phr2-polh	100	100	100	100	100	93.7	11.6

Table 3

Percent mortality of *H. armigera* larvae infected with recombinant OBs kept in the dark or illuminated with visible light after UV irradiation at a dose of 2460 J/m^2

Virus	UV dose (J/m^2)			Mean mortality % (SD)
	0	2460	2460 [a]	
Hear-polh (dark)	100	25.0	37.5	31.3 (8.8)
Hear-polh (light)	100	31.9	21.0	26.5 (7.7)
Hear-phr2-polh (dark)	100	10.4	37.5	24.0 (19.2)
Hear-phr2-polh (light)	100	93.8	82.2	88.0 (8.2)

[a] Repeated bioassay

4. Discussion

It has been reported that the inactivation rate of both wild type and recombinant HearNPVs (HaSNPV-EGT and HaSNPV-AaIT), positively correlated with the dose of UV irradiation (Sun et al., 2004a). DNA damage is likely to be a major reason for UV inactivation of these and other baculoviruses. Therefore UV protectants are being used to protect the baculovirus from irradiation decay. One of two DNA photolyase genes present in the genome of ChchNPV (*Cc-phr2*) encodes a functional DNA repair enzyme (van Oers et al., 2008). So far no information is available whether PHR2 also functions *in vivo* and whether it has anything to do with DNA repair from UV inactivation. Incorporation of *Cc-phr2* in HearNPV, which does not contain DNA photolyases genes, is a way to test whether Cc-PHR2 has *in vivo* activity in baculovirus biology.

The results obtained in the current study supported the hypothesis that PHR2 can protect a virus against UV irradiation. OBs of a recombinant virus that expressed *Cc-phr2* (Hear-phr2-polh) and which were treated with visible light after exposure to UV irradiation, induced an approximately 3-fold higher mortality in *H. armigera* larvae than OBs that were kept in the dark after UV irradiation or

than OBs from a HearNPV that did not contain a *phr2* gene (Hear-polh) (Table 3). This result suggests that the presence and expression of *Cc-phr2* was able to provide a higher level of UV resistance to HearNPV and most likely Cc-PHR2 was responsible for this effect by repairing DNA. This result was obtained despite the fact that only a very small proportion of the viral genome copies in the virus samples used for the bioassay (0.2%) indeed contained *Cc-phr2*. This may also indicate that a very low amount of Cc-PHR2 is sufficient to repair damaged DNA. This is in line with the finding that the abundance of native CPD photolyases is very low in *E. coli,* which contains only 10 to 20 active molecules per cell in the stationary phase (Alcorn and Rupert, 1990).

PCR analyses demonstrated that the OB stocks of both recombinant, bacmid-derived HearNPVs contained genomes similar to wild-type HearNPV (Fig. 3) and that these occurred as soon as BVs were generated in bacmid-transfected HaAM1 cells. Therefore, the HzAM1 cell line was examined for the presence of latent wild type HearNPV by PCR, but it did not contain a contamination with wild-type virus above the detection level (data not shown). These studies indicated that the generation of wild-type HearNPV might not have been caused by activation of a latent infection in the host, but is most likely explained by an intrinsic instability of the recombinant bacmid genomes (Hear-polh and Hear-phr2-polh). Such instabilities have been noted before in cells infected with AcMNPV bacmids and contributed to the so-called 'passage effect' (Pijlman et al., 2003b).

The inserts in Hear-phr2-polh might be deleted by intramolecular genomic recombination between homologous sequences (Fig. 4). The HearNPV *polh* promoter, the first 258 nucleotides and the last 31 nucleotides of the *polh* ORF and its downstream poly-A region were found to be present in the HaBacHZ8 bacmid by sequencing this part of the bacmid (sequenced by Dr. Marcel Westenberg). However, these sequences were also present in the expression cassette inserted into the two recombinant HearNPVs used in this study, which contained the complete HearNPV *polyhedrin* gene to generate OBs. These homologous *polyhedrin* gene regions had sufficient length to facilitate recombination between individual bacmid molecules, thereby removing the entire bacterial cassette, including the *phr* ORF and resulting in a wild-type HearNPV genome (Fig. 4). The recombination rate was quite high or competition was very strong against the bacmid insertions as, on the basis of qPCR, almost 96% of the viral genomes were wild-type after three rounds of propagation in insect cells followed by one passage in larvae.

Approximately 7% of recombinant viruses in which a bacterial cassette was retained, also contained *Cc-phr2,* indicating selective loss of this gene from the cassette independent from the loss of the whole cassette as a consequence of recombination. The hypothesis behind this observation may be that the overexpressed Cc-*phr2* in HearNPV might be toxic for the cells or prevent viral replication by competing for DNA binding with other proteins involved in processes such as DNA replication and transcription. It has recently been shown that baculovirus Cc-PHR2 photolyase has binding capacity to chromosomal DNA (Xu et al., 2010).

The observation that OBs of Hear-phr2-polh are more resistant against UV inactivation than OBs of the Hear-polh control virus when given the possibility for photoreactivation by exposure to visible light after UV irradiation, provides a first indication that *Cc-phr2* might be active when inserted into a *phr*-negative background. Since the photoreactivation occurs with OBs, it must be assumed that the Cc-PHR2 proteins are packaged into ODVs. When the DNA photolyase is packaged into the ODV, it may directly repair the CPDs already in the OBs. This would be more effective than when *phr* genes would need to be expressed first after entry of the virus into the cells, as the *phr* genes itself may be inactivated by CPDs. However, whether the Cc-PHR2 was indeed present in the ODVs of recombinant viruses encoding the PHR2 protein proved difficult to determine as the levels of PHR2

peptides in LC-MS/MS were below the detection limit (data not shown). The low proportion of Hear-phr2-polh recombinants in the virus stock may have complicated this mass-spec analysis.

In conclusion, a recombinant HearNPV (Hear-phr2-polh) carrying the ChchNPV *phr2* ORF was constructed and was shown to reduce the UV sensitivity, even though a substantial amount of recombinants lost the *Cc-phr2* coding sequence, probably due to a combination of recombination events and counter selection because of putative toxic affects of overexpressed Cc-*phr2*. To improve the stability of bacmid-derived, recombinant HearNPV genomes in general, the *polh* gene regions remaining in the HaBacHZ8 bacmid need to be removed. The *Cc-phr2* insert may be further stabilized by insertion of an additional *hr* sequence, as has been done for AcMNPV (Pijlman et al., 2004). When more homogeneous Hear-phr2-polh virus is available, bioassays need to be repeated to show rescue from UV irradiation, as well as the LC-MS/MS experiments to demonstrate the presence of Cc-PHR2 in the recombinant HearNPV ODVs independently. Addition of a UV resistance gene into baculoviruses might be a major step forward in commercialization of baculovirus insecticides provided genetically modified baculoviruses are accepted by the market as a biocontrol agent for cotton bollworm.
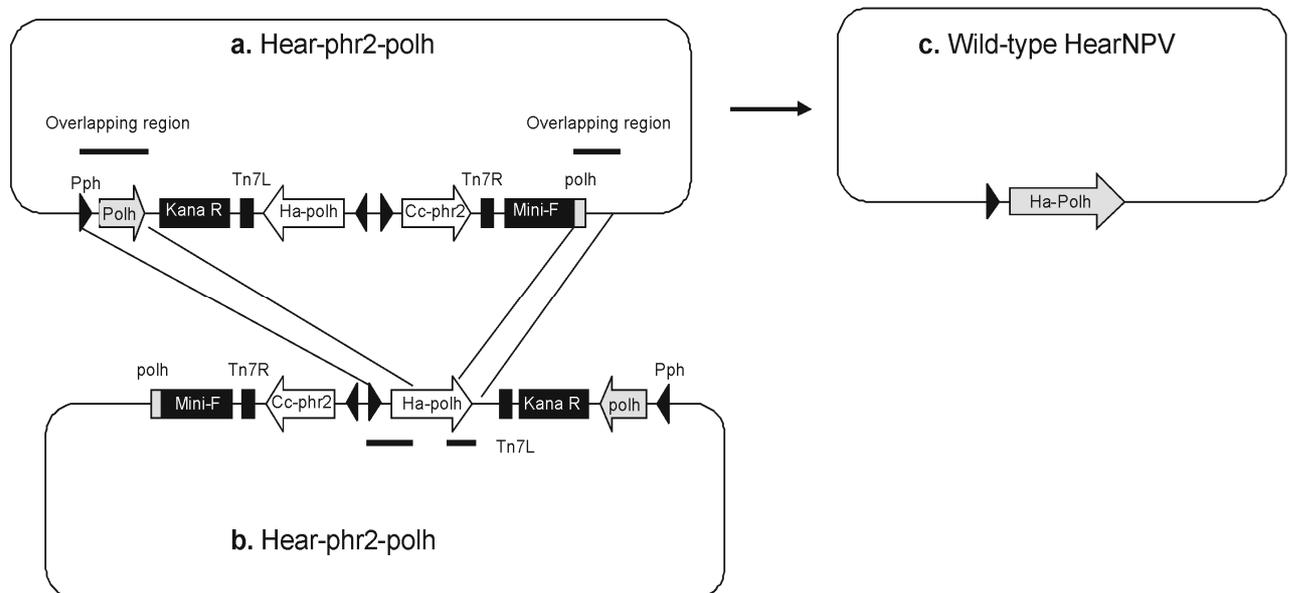

Figure 4. Schematic representation of genomic recombination between two HaBacHZ8-derived bacmids in insect cells. Two Hear-phr2-polh bacmids (a & b) are given in opposite orientation recombine (indicated with bars) at regions presented twice in each genome: the HearNPV *polh* promoter and the first 258 nucleotides of the *polh* ORF and the last 31 nt of the ORF and the downstream poly-A region. As a consequence, the entire bacterial cassette with the transposed inserts is deleted to produce a wild-type HearNPV genome (c).

Acknowledgments

Chapter 6

Establishment of a cell line from *Chrysodeixis chalcites* permissive for *Chrysodeixis chalcites* and *Trichoplusia ni* nucleopolyhedrovirus

Abstract

A new cell line was established from the embryos of the insect *Chrysodeixis chalcites* (Lepidoptera, Noctuidae, Plusiinae). The cell line contains several morphologically different cell types and was distinguished from three other lepidopteran cell lines propagated in the laboratory by DNA amplification fingerprinting. The cultured cells, which we officially named WU-CcE-1 cells, were permissive for infection by *C. chalcites* nucleopolyhedrovirus (ChchNPV) and large numbers of occlusion bodies were produced that retained their infectivity for *C. chalcites* larvae. The CcE-1 cells were also permissive for *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV). ChchNPV could be passaged in these cells for at least four passages indicating that budded virus production was supported. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and Helicoverpa armigera *(Hear) NPV* both induced apoptosis in these cells. The results obtained indicate that the CcE-1 cell line will be a useful tool in the study of both ChchNPV and TnSNPV.

Keywords: cell line, *Chrysodeixis chalcites*, ChchNPV, DNA fingerprinting, baculovirus

1. Introduction

Invertebrate cell cultures are commonly used in a wide range of studies, which focus on insect physiology, developmental biology, and pathology. The first paper on this topic was published in the *Chinese Science Bulletin* in 1958, and reported that Gao and his associates had established the first continuous insect cell line, which was derived from the silkworm, *Bombyx mori* (Gaw, 1958; Vlak, 2007). Later in 1962, Grace successfully established cell lines from ovaries of *Antheraea eucalypti* (Grace, 1962). Since then, over 500 continuously growing cells lines have been obtained from over 100 insect species (Lynn, 1999).

For baculovirus research these insect cell lines are important tools and they are also a means for large scale production of baculovirus-based insecticides (Black et al., 1997). In addition, numerous proteins of interest for medicine and biology have been produced in cultured insect cells as a consequence of the development of the baculovirus expression system (see for instance Beljelarskaya, 2002; Hitchman et al., 2009; van Oers, 2006 for reviews). The first vaccine for human use on the market, that is produced in insect cells, is directed against human papilloma virus, the causative agent of cervical cancer (see Harper (2009) for a review). Currently, insect cells are also being used for the production of gene therapy vectors such as adeno-associated virus vectors (Urabe et al., 2002) and baculovirus-based gene delivery vectors (Hu, 2006).

The tomato looper *Chrysodeixis chalcites* (Lepidoptera, Noctuidae, Plusiinae) is a polyphageous insect. The species is endemic to tropical Africa and Southern-Europe, and is a common pest in sweet pepper, tomato and ornamental flowers in Dutch green houses. A nucleopolyhedrovirus (NPV) has been isolated from *C. chalcites* larvae from a greenhouse in Naaldwijk, the Netherlands (van Oers et al., 2004). This virus isolate, ChchNPV-NL, belongs to group II NPVs and has singly-enveloped nucleocapsids (SNPV). Its whole genome sequence was analyzed revealing a unique feature, the presence of two cyclobutane DNA photolyase genes (van Oers et al., 2005; van Oers et al., 2008). These genes are potentially involved in repairing UV damage in DNA.

To study ChchNPV-pathology in further detail and to determine whether the DNA photolyase genes play a role in protecting ChchNPV against UV damage, a cell line susceptible for ChchNPV is highly desirable. Such a system would facilitate the production of mutant viruses and recombinants by applying, for instance, bacmid technology (Luckow et al., 1993). In a previous study, the susceptibility of *Trichoplusia ni* High Five cells (Granados et al., 1994) to ChchNPV was examined by inoculation with ChchNPV-containing hemolymph. The cells showed little cytopathological effects (CPE) at 4 d p.i. and a very low percentage (<1%) of the cells accumulated viral occlusion bodies (OBs) or polyhedra (van Oers et al., 2004). With these cells we were not able to establish a second round of infection with medium collected from this first passage, suggesting that no infective budded virus particles were released. Other *T. ni*-derived cell lines such as Tn368 performed even worse (unpublished data). Similar data have been obtained by others recently when trying to infect *T. ni*-derived cell lines with *Trichoplusia ni* (Tn) SNPV (M. Erlandson, personal communication), a virus closely related to ChchNPV.

These data suggest that the *T. ni* High Five cell line was only semi-permissive to ChchNPV and not an effective tool for the further study of ChchNPV. Therefore, a new cell line derived from *C. chalcites* embryos was established and characterized in this study. The novel cell line was tested for its susceptibility to ChchNPV and the ability to support the production of infectious budded virus (BV) and occlusion derived virus (ODV). Electron microscopy analysis was applied to follow the cytopathology of ChchNPV over time. The collective data indicate that the *C. chalcites* cell line

supports replication of both ChchNPV and TnSNPV and represent a novel tool to study these and possibly other viruses that infect plusiine insects.

2. Methods and materials

2.1. Cell line development and maintenance

Pupae of a Spanish biotype of *C. chalcites* were collected in the field and used to establish a culture of *C. chalcites* insects in the laboratory as described before (van Oers et al., 2004). A primary cell culture was developed from embryos from these insects based on previously described procedures (Lynn, 1996). The culture medium used in this study is Sf-900 II SFM (Invitrogen) supplemented with 5% fetal bovine serum (*Seromed*). Gentamycin (Invitrogen) was added to a final concentration of 50 µg/ml. Briefly, approximately thirty 2-day old *C. chalcites* eggs were rinsed twice with sterile distilled water and disinfected in 70% ethanol for 5 min. After two additional rinses with water, eggs were disrupted to release the embryos in cell culture medium. Embryonic tissue was recovered with 0.1- 0.2 ml culture medium and kept in a tightly sealed 35 mm Petri dish (Nunc) at 27 ºC. After 24 h incubation, an additional 1 ml medium was added to the dish. During the initial period, additional fresh culture medium (about 0.5 ml every 3 days) was added to the cells. When the Petri dish contained about 3 ml medium, 2.5 ml was replaced with 0.5 ml fresh medium. This process of adding and replacing medium continued for 4 weeks before a substantial number of viable cells were available for subculturing. Cells that grew from the primary cultures were subcultured by trypsinization and maintained in culture medium as described above.

2.2. Identification of cell lines

Characterization of the *C. chalcites* cell line, designated WU-CcE-1 (further referred to as CcE-1 in this paper), was carried out using the DNA amplification fingerprinting technique as described (McIntosh et al., 1996). Total cellular DNA was extracted from CcE-1 cells and from the three cell lines maintained in the laboratory during CcE-1 cell line preparation, *T. ni* High Five (Granados et al., 1994), Sf9 (Vaughn et al., 1977) and HzAM1 (McIntosh and Ignoffo, 1981). The Qiagen Tissue DNA purification kit was used to extract cellular DNA from 2×10^6 cells from each cell line. Three pairs of primers were used in this study as described in McIntosh et al. (1996). These primers were originally designed for PCR amplification of the mammalian aldolase, prolactin receptor and interleukin-1β sequences: Primer set 1 (aldolase): 5'-CCGGAGCAGAAGAAGGAGCT-3' and 5'-CACATACTGGCAGCGCTTCA-3', primer set 2 (prolactin receptor) 5'-CTGGGACAG-ATGGAGGACT-3' and 5'-CTCAGGTTTTAATCGAATTT-3', and primer set 3 (interleukin-1β) 5'-ATGAGGATGACTTGTTCTTT-3' and 5'-GAGGTGCTGATGTACCAGTT-3'. Each PCR was performed in a volume of 50 µl containing 300 ng genomic DNA, 10 µl PCR reaction buffer (Promega), 15 pmol of both primers, 3 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen) and 5 units Taq DNA polymerase (Promega). PCR was performed with an initial denaturation step at 95 ºC for 3 min, followed by 40 cycles of 95 ºC for 5 s, 40 ºC for 1 s, 72 ºC for 30 s, and a final extension step at 72 ºC for 5 min. PCR products were examined by electrophoresis in a 2.5% agarose gel in the presence of ethidium bromide.

To further confirm the origin of the CcE-1 cells, a set of degenerate *phr*-primers (5'-CGAAAACTGTTTATATCGAAGG-3' and 5'-CAGGAAACAGCTATGACCDATYTTYTTN-GCCCARTA-3') (Xu et al., 2008) was used to partially PCR amplify the cDNA of the insect *phr*

transcripts of the CcE-1 cells. cDNA was made as described before (van Oers et al., 2004) and the product was sequenced.

2.3. Baculovirus infection studies

The baculoviruses used in this study included *Autographa californica* (Ac) MNPV strain E2 (Vail et al., 1971), ChchNPV-NL (van Oers et al., 2004), TnSNPV (Willis et al., 2005) and *Helicoverpa armigera* (Hear) NPV-G4 (Sun et al., 1998). For initial infections hemolymph with ChchNPV was collected from infected *C. chalcites* larvae. TnSNPV-containing hemolymph was obtained from infected *T. ni* larvae, and kindly provided by M. Erlandson (Saskatoon Research Centre, Agriculture and Agri-Food Canada). AcMNPV and HearNPV inocula were derived from infected Sf9 and HzAM1cells, respectively. To verify whether AcMNPV and HearNPV were able to enter CcE-1 cells, bacmid-derived AcMNPV carrying a *gfp* reporter gene and an *egfp*-containing HearNPV bacmid were used. The *gfp* ORF was cloned downstream of the AcMNPV IE1 promoter in the AcMNPV bacmid (constructed by Dr. Salvador Herrero at Wageningen University). The *egfp* ORF was introduced into HaBacHZ8 under control of the AcMNPV p10 promoter together with the polyhedrin gene to generate HaBac-*egfp-ph* (Song et al., 2008).

To screen for virus susceptibility, CcE-1 cells (5 x 10^3 cells) were seeded in 6-well tissue culture plates (Nunc) in 1 ml culture medium following the routine subculture procedure. CcE-1 cells were inoculated with 50 µl hemolymph or 80 µl of virus-containing cell culture medium, respectively, in a total volume of 1 ml medium. One ml fresh medium was added to the cells after 2 h incubation at 27 ℃. The cell cultures were examined for 6 days by phase contrast microscopy for the occurrence of CPE and with an epifluorescent microscope for the presence of (E)GFP. At 6 days p.i. the medium was collected from the ChchNPV-inoculated wells, which showed a positive CPE. One hundred microliters of the filtered medium were used to re-inoculate approximately 60% confluent 25 cm^2 CcE-1 cell cultures for three rounds of infection. The medium collected from TnSNPV-infected CcE-1 cells was passaged two times in a similar way.

2.4. Identification of progeny virus from CcE-1 cells by PCR

To identify the progeny virus produced in the CcE-1 cells, the BV-containing supernatant was collected by centrifugation at 2000 x *g* (Heraeus Labofuge I) for 5 min. Viral DNA was extracted and served as a template for the following PCR analysis. The primers (5'- CATGGTACCACGATGGCAGCAAACGATCG-3') and (5'- GTCCGGCCGCATTTGTTATT-AATAATGTAT-3') were used to detect the *Cc-phr2* gene in ChchNPV. A set of degenerate *phr*-primers as described above was used to partially amplify the TnSNPV *phr* gene (Willis et al., 2005). The identity of each PCR product was verified by direct sequence analysis.

2.5. Protein analysis

Infection of CcE-1 cells in 6-well tissue culture plates was carried out with 40 µl medium containing ChchNPV BV, which was obtained from the third viral passage over CcE-1 cells. Cells were incubated at 27 ℃, harvested by physical deattachment and collected by centrifugation (Heraeus Labofuge I) at 2000 x *g* for 5 min at 1, 2, 3, 4 or 6 d p.i. The CPE at each time point was also examined microscopically prior to harvesting. After two rinses with PBS, total cell extracts were prepared in Laemmli buffer and equal volumes of each sample were separated by 4-15% gradient SDS-PAGE (Bio-Rad). One gel was stained with Coomassie Brilliant Blue, while a duplicate gel was subjected to immuno-detection of the very late polyhedrin protein (Rohrmann, 1986) To this aim,

rabbit polyclonal antibodies against AcMNPV polyhedrin (diluted 1:4000, made by E. Roode, Laboratory of Virology, Wageningen, The Netherlands) were used. The procedure for western blot analysis was the same as previously described (Xu et al., 2010).

2.6. Electron microscopy analysis

The procedures for the infection with ChchNPV BV and the harvesting of the infected CcE-1 cells were the same as mentioned above for protein analysis. The method used to fix and embed the cells for EM was adapted from van Lent et al. (1990). Ten thousand cells were fixed for 1 h at room temperature with 2% paraformaldehyde and 3% glutaraldehyde in PC-buffer (0.1 M Na_2HPO_4, 9.7 mM citric acid, pH 7.2, 1.5 mM $CaCl_2$). Cells were collected by centrifugation at 2,000 *g*, washed twice in PC-buffer and resuspended in 5% (w/v) gelatine in PC-buffer at 37 °C. Cells were pelleted by centrifugation for 10 s in an Eppendorf centrifuge at 16,000 x *g*. Excess gelatine was removed and the pellet was hardened by cooling on ice. The gelatine blocks containing a high concentration of cells were cut into small pieces and subsequently treated with the aldehyde fixative described above for 15 min at RT. The specimens were washed with PC-buffer, further fixed for 1 h at room temperature with 1% (w/v) OsO_4 in PC-buffer. After three times washing with water, the specimens were dehydrated in a series of increasing concentrations of ethanol and embedded in LR White resin (London Resin Company). Ultrathin sections of 60 nm were cut with a diamond knife using a Leica Ultracut S microtome. Sections were collected on 100 mesh copper grids supplied with a formvar film. Sections were stained for 5 min with 2% uranyl acetate and 10 min with lead citrate (Reynolds, 1963). Sections were then examined in a JEOL JEM2100 microscope at 120 kV and digital micrographs were made with a Gatan US4000 camera.

2.7. OB infectivity assay

C. chalcites larvae were fed with OBs purified from ChchNPV-infected CcE-1 cells at 9 d p.i. To this aim, second instar larvae were starved for at least 8 h at 26 °C prior to infection. The starved larvae were fed by droplet feeding with an OB suspension at concentrations of 10^2 OBs/ml, 10^4 OBs/ml and 10^6 OBs/ml, respectively, following a previously described procedure (Hughes and Wood, 1981). The virus suspension was made in 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue. For each OB concentration, 24 larvae were inoculated and incubated at 26 °C. Mortality was checked every 24 h until death or pupation of the larvae.

3. Results and discussion

3.1. Cell growth and morphology

Cells derived from *C. chalcites* embryos initially migrated from the pieces of embryo tissue and attached to the bottom of the culture dish and multiplied gradually for several weeks. During this initial period, the cell line consisted mostly of small spherical cells and some fibroblast-like cells, loosely adhering to each other (Fig. 1A). The cells were subcultured and a continuous cell line gradually developed from the primary cultures. After eight passages, the subculture periods became shorter, changing from several weeks in the beginning to regular intervals of 6-10 days. Compared to the parental cells, the newly established cell line (WU-CcE-1) firmly attached to the bottom of the culture flask and could only be released by trypsin treatment. Around passage 8-10 individual cells exhibited different morphologies and spherical cells, epithelial-like cells, fibroblast-like cells and some myoblast-like cells were observed, probably as a consequence of cell differentiation. Cells with

similar morphology aggregated to form colonies. Around 70% of the cells displayed a fibroblast-like morphology after 15 passages (Fig. 1B). The doubling time of CcE-1 cells was measured at passage 33 and was 28 h. CcE-1 cells at passage 10 were used for further characterization.
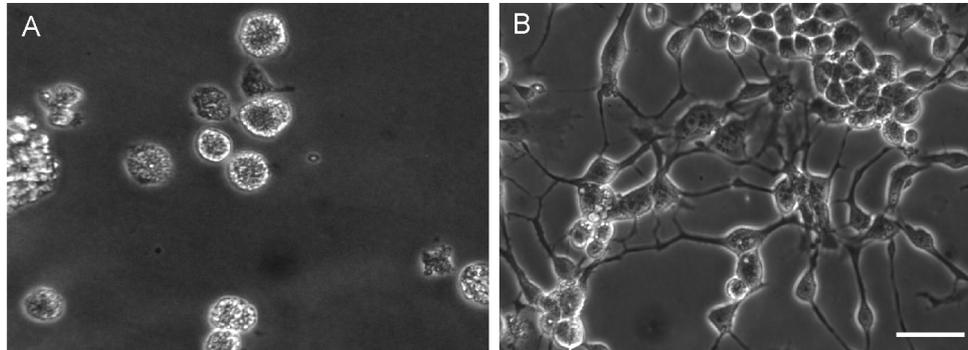

Figure 1. Phase contrast micrographs of the *Chrysodeixis chalcites* cell line. (A) Primary cell culture; (B) 15th passage of CcE-1 cells. Both micrographs are taken at the same magnification. The marker bar is 40 μm.

3.2. Cell line characterization by DAF

DNA amplification fingerprinting is a reliable method for identification of insect cell lines and has previously revealed mislabeling and contamination of arthropod cell lines (McIntosh et al., 1996). The DNA fingerprint of the CcE-1 cell line and three other lepidopteran cell lines was generated by PCR amplification using three different primer sets developed for human cell lines: for aldolase (1), for the prolactin receptor (2) and for interleukin-1β (3). The molecular size of the major PCR products visualized in agarose gels was in the range of 80- 2000 bp (Fig. 2). The DNA fingerprint profiles illustrated that CcE-1, *T. ni* High Five, Sf9 and HzAM1 cell lines, all grown simultaneously in the laboratory, could be individually distinguished with primer set 1 (aldolase) by the number, the size and the quantity of the detected products (Fig. 2A). DNA fingerprinting profiles obtained with the other two primer sets also revealed that the cell lines co-currently grown in the laboratory are clearly distinguishable from each other (Fig. 2B and C). Furthermore, the partial CPD Class II photolyase cDNA sequence of CcE-1 cells was analyzed and compared with that of *C. chalcites* larvae obtained with the same degenerate primers (manuscript in preparation). The fact that this gave the same nucleotide sequence for cells and larvae is further confirmation that the CcE-1 cells indeed originated from *C. chalcites.*

3.3. Susceptibility to ChchNPV

ChchNPV BV-containing hemolymph was used to inoculate CcE-1 cells giving a typical CPE (data not shown). A time course of the infection initiated with virus passaged three times in CcE-1 cells is shown in Fig. 3. The CPE was seen in some cells as early as 1 d p.i. and a number of cells had produced OBs at 2 d p.i. At 6 d p.i., nearly all the cells were showing CPEs and were filled with OBs (Fig. 3). The infection pattern with passage three looked similar as that obtained with the ChchNPV-containing hemolymph. The successful infection demonstrated that BVs derived from CcE-1 cells were still infectious and that the virus replicated at least till passage four. The titer of the produced ChchNPV increased over the passages and had a titer of 3.1 x 10^7 TCID$_{50}$/ml after three passages.
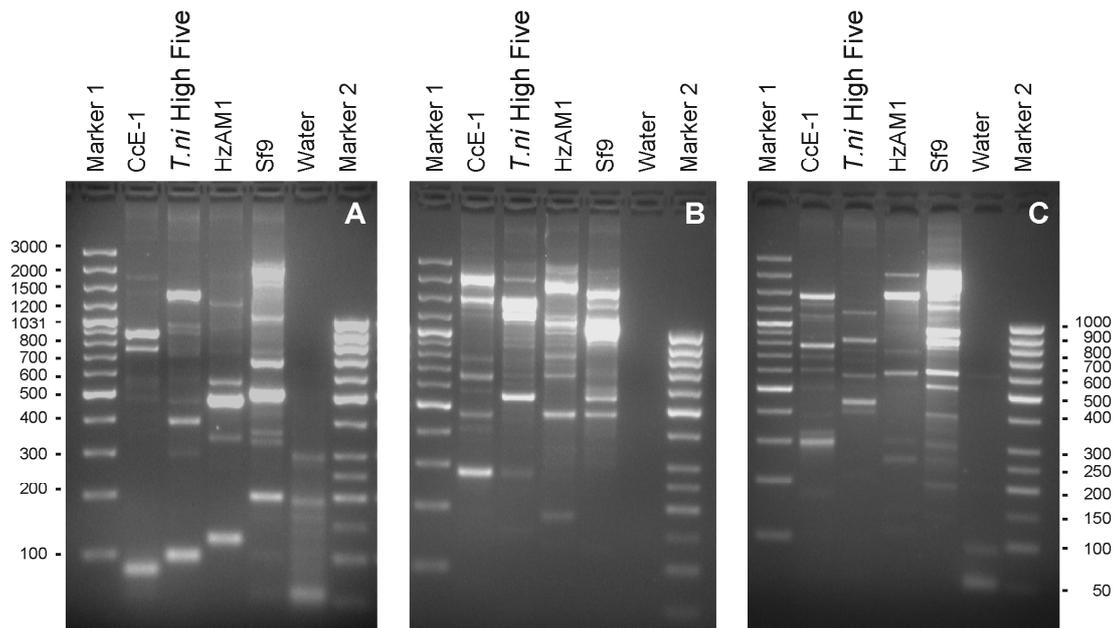
Figure 2. DAF profiles of CcE-1, *T. ni* High Five, Sf9 and HzAM1 cell lines with primer set 1 (A); with primer set 2 (B); and primer set 3 (C); water served as negative control in place of DNA. GeneRuler 100 bp 50 bp DNA ladders (Fermentas) were used as size markers and are indicated on the sides in bp.

To verify that the virus produced in CcE-1 cells is indeed ChchNPV, DNA was extracted from the BVs harvested from passage three and subjected to PCR to amplify one of the viral photolyase genes (*Cc-phr2*), which is a typical feature for ChchNPV. The virus was identified by a clear 1.5 kbp PCR product (data not shown), and its sequence was confirmed by direct sequencing to match with the *Cc-phr2* gene. In addition, proteomic analysis of ChchNPV BVs derived from the CcE-1 cells identified a collection of peptides of ChchNPV BV associated proteins, including the F protein, which matched with the ChchNPV ORF database (manuscript in preparation).

Protein samples of ChchNPV-infected CcE-1 cells collected at different time points were analyzed by SDS-PAGE (Fig. 4). To follow the timing of polyhedrin synthesis Western blot analysis was performed and the polyhedrin protein (approximately 30 kDa), was observed from 2 to 6 d p.i. and accumulated over time, showing that, as expected, the AcMNPV antibody cross-reacted with the ChchNPV polyhedrin. Polypeptides smaller that 30 kDa were also observed, probably as the consequence of protein degradation. The very late P10 protein (reviewed by van Oers and Vlak, 1997) was also observed in the Coomassie blue stained gels from 3 d p.i. (data not shown) and also accumulated over time, in line with the appearance of fibrillar structures as seen in the EM pictures (see Fig. 5).
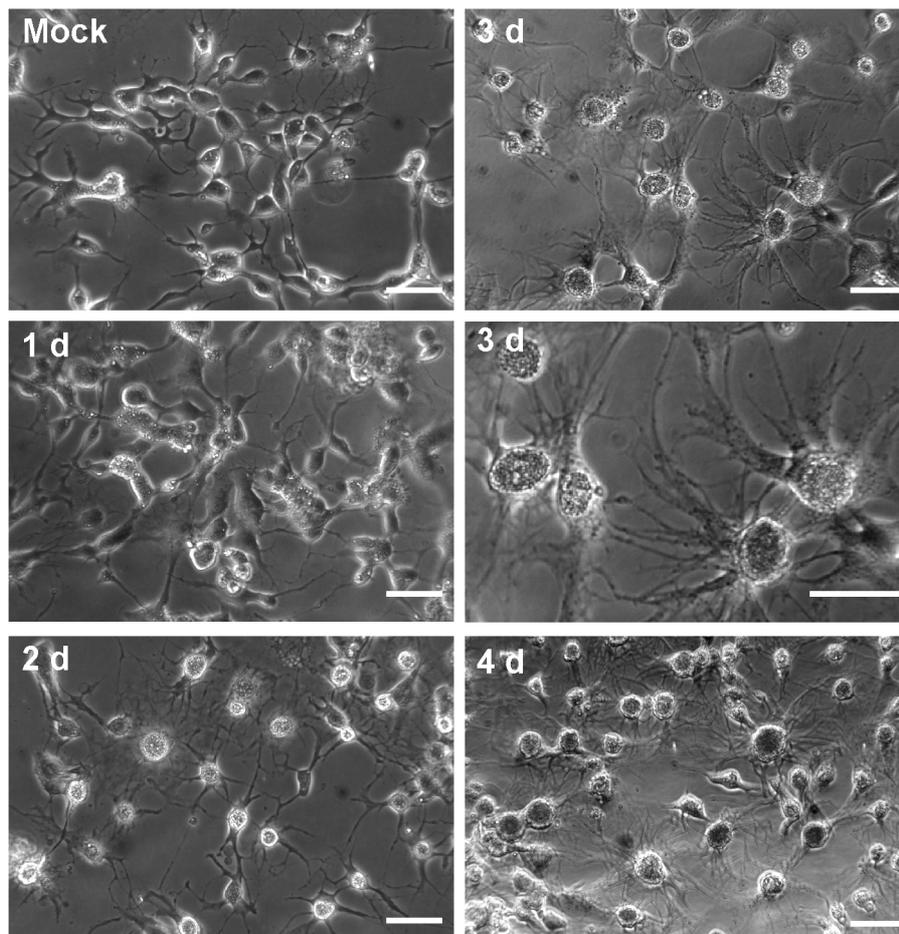
Figure 3. Time course phase contrast micrographs of CcE-1 cells inoculated with ChchNPV. CcE-1 cells were infected with 40 μl budded virus passaged three times in cell culture. Two magnifications are given for the 3 d p.i. time point. The marker bars indicate 40 μm.
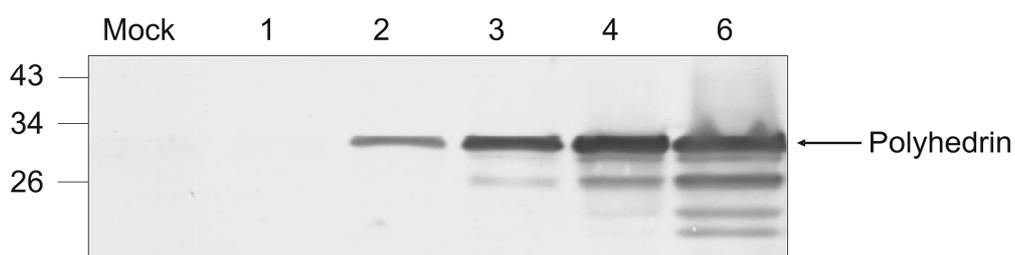
Figure 4. Detection of polyhedrin in ChchNPV infected CcE-1 cells over time. Protein synthesis was followed from 1-6 d p.i. in a Coomassie stained SDS-PAGE gel and the polyhedrin protein was detected with antiserum directed against AcMNPV polyhedrin by western blot analysis. A size marker in kDa is indicated on the left.

The cytopathology of ChchNPV in CcE-1 cells was also studied by electron microscopy from 1 to 6 d p.i. (Fig. 5). Nucleocapsids were observed in the nucleus of cells and they assembled into virus particles to become singly-enveloped occlusion-derived virons (ODVs). By 24 h p.i., almost all the cells contained both nucleocapsids and virus particles. OBs assembled and ODVs were being occluded at 24 h p.i. Fibrillar structures were observed in both the cytoplasm and the nucleus (Fig. 5B, C and D). At 48 h p.i. the amplification of ChchNPV and the production of progeny virus had

intensified. Fibrillar structures associated with electron-dense spacers and virogenic stroma were observed and the stroma was surrounded by OBs as described previously for other baculoviruses (Granados and Lawler, 1981; Vlak et al., 1988). At 48 h more OBs were produced, their size had increased and the OBs contained virus particles. OBs were also frequently surrounded by an outer 'electron-dense' layer (Fig. 5D and E), the polyhedral envelope, as observed before for related viruses (Miller, 1997b). At 6 d p.i. (Fig. 5F), the nucleus was packed with OBs, virogenic stroma and virus particles, and a large number of OBs were liberated from the cells. All these data suggest that ChchNPV infection in CcE-1 cells induces the CPE common for baculovirus infections, that OBs with progeny virus were assembled and that the infection was fully permissive. The CcE-1 cells recovered from the liquid nitrogen stock made at passage 15 were still highly permissive for the infection with ChchNPV.
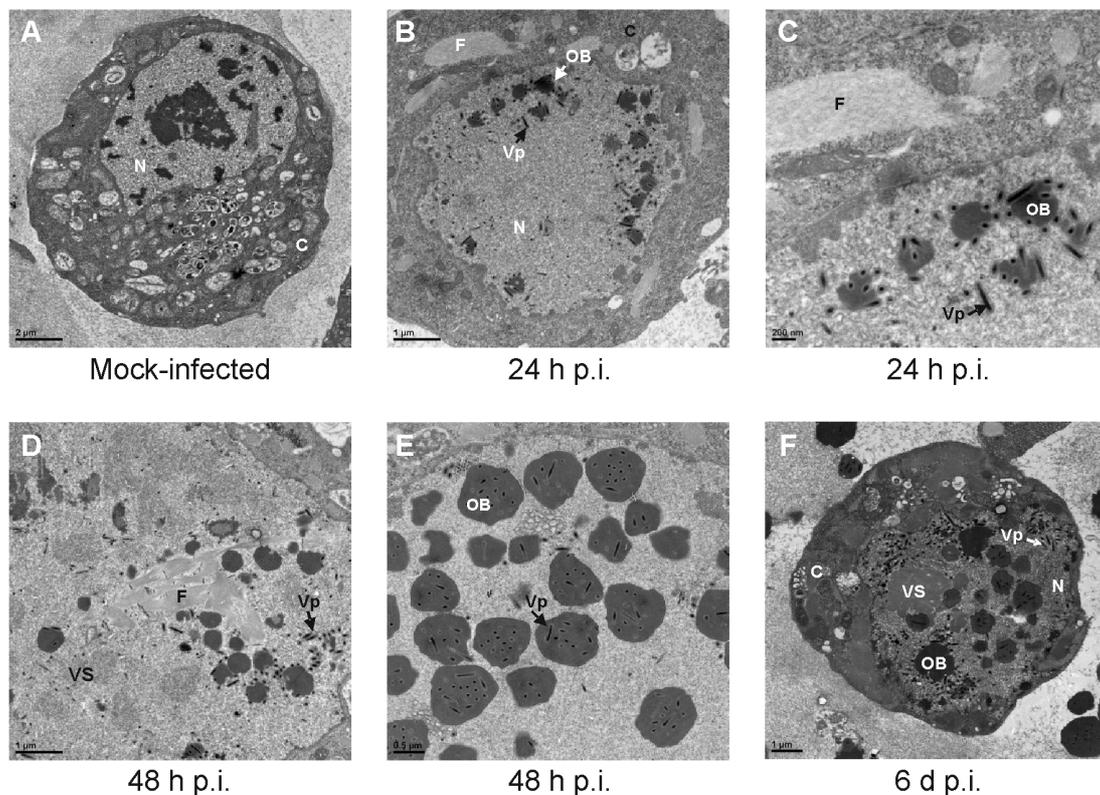
Figure 5. Electron micrographs of thin sections of CcE-1 cells infected with ChchNPV. Healthy cells (A) and infected cells were harvested at 24 h p.i (B and C), 48 h p.i. (D and E), and in the very late stage of infection (6 d p.i.) (F) and examined at various magnifications. Marker bars are shown on each micrograph. Virogenic stroma (VS), occlusion body (OB), virus particles (Vp) and fibrillar structures (F) are indicated, as well as the nucleus (N) and cytoplasm (C).

3.4. Infectivity of OBs to the insect

To fully characterize the properties of the cell line and assess its applicability it is important to know whether the OBs produced in the cells cause infection in insects. A bioassay was carried out in *C. chalcites* larvae with OBs derived from CcE-1 infected cells. All larvae that were droplet fed with a suspension of 10^6 OBs/ml died and showed the typical symptoms of polyhedrosis. All larvae treated with the lowest concentration (10^2 OB/ml) survived, and 20% mortality was obtained with 10^4

OBs/ml. Control larvae survived and developed normally. These results showed that the OBs of ChchNPV derived from CcE-1 cells are indeed infectious for larvae.

3.5. Susceptibility to other NPVs

The susceptibility of the cells for other baculoviruses (AcMNPV, HearNPV and TnSNPV) was also tested. The CcE-1 cells were also permissive to TnSNPV with a clear CPE and a large quantity of OBs formed (Fig. 6). Compared to the replication of ChchNPV in this CcE-1 cell line, the appearance of the CPE and the OBs with TnSNPV infection seemed to take longer (3 days before the first OBs appeared instead of two). However, the unknown multiplicity of infection with hemolymph-derived ChchNPV and TnSNPV in CcE-1 cells complicates the comparison of the replication rate between these two virus species. Successful, successive infections with TnSNPV-containing medium demonstrated that TnSNPV budded from CcE-1 cells was infectious over at least three passages. The nature of TnSNPV BV derived from CcE-1 cells was confirmed by PCR analysis.

CcE-1 cells did not show any sign of successful infection with either AcMNPV or HearNPV. Both NPVs induced apoptosis in these cells (Fig. 6), as judged from characteristic morphological features of cell shrinkage, nuclear fragmentation and apoptotic bodies (Clem et al., 1991). AcMNPV and HearNPV carried a *(e)gfp*-marker under an early and a very late promoter, respectively. Green fluorescence was observed in the cells treated with both *(e)gfp*-carrying NPVs at 24 h p.i. and cells started to go into apoptosis afterwards (data not shown). This result indicates that both AcMNPV and HearNPV are able to enter CcE-1 cells and can express E(GFP), but that these viruses did not successfully replicate in these cells due to apoptosis. The cellular apoptotic response serves as a defense against baculovirus infection. Apoptosis negatively affects viral pathogenesis and reduces the production of progeny viruses (Clem, 2005). Specific viral gene products, such as the caspase inhibitor (Clem et al., 1991) and inhibitors of apoptosis (IAPs) (Chen et al., 2000a), are known to block the apoptotic response. In this study, the infection with AcMNPV or HearNPV triggered apoptosis in CcE-1 cells. Therefore, the apoptosis inhibitors encoded by these two viruses, are apparently not able to block apoptosis pathways in CcE-1 cells. *T. ni* cells on the other hand do not go into apoptosis after AcMNPV infection, even when the *p35* gene is deleted (Clem et al., 1991; Hershberger et al., 1992).

Previous studies revealed that both ChchNPV and TnSNPV belong to group II NPVs and infect noctuids in the subfamily Plusiinae (Herniou and Jehle, 2007; Xu et al., 2008). Their genomes are highly collinear (van Oers et al., 2005; Willis et al., 2005) and this may result in an at least partially overlapping host range. This could explain why the CcE-1 cells are susceptible to both ChchNPV and TnSNPV, but not to AcMNPV or HearNPV.

4. Conclusions

In the current study, we developed the first cell line from the tomato looper *Chrysodeixis chalcites*. The CcE-1 cell line supports replication of both ChchNPV and TnSNPV. This novel cell line will be a useful tool for the generation of a ChchNPV bacmid and the production of recombinant viruses. Since the current *T. ni* High Five cell line is not able to support successful replication of TnSNPV, the CcE-1 cells will be very attractive for studying TnSNPV as well.

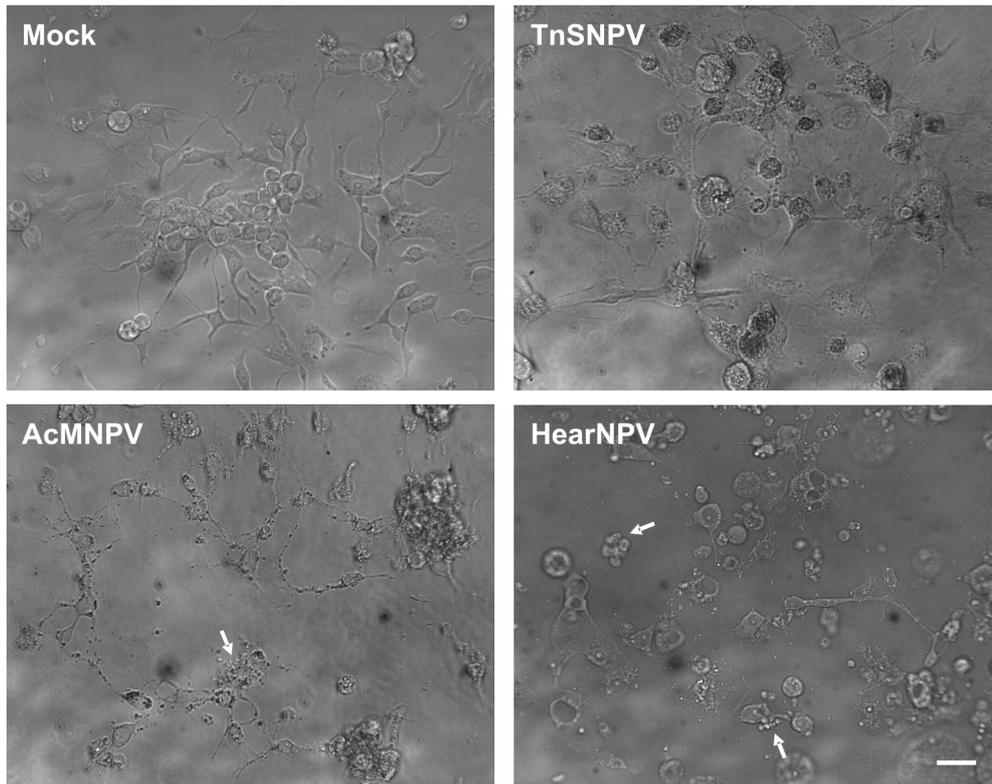**Figure 6.** Phase contrast micrographs of CcE-1 cells inoculated with the heterologous viruses, TnSNPV, AcMNPV and HearNPV, respectively. The micrograph of TnSNPV inoculated cells was taken at 6 day post inoculation, while those of AcMNPV and HearNPV inoculated cells were taken at the third day. All micrographs were taken at the same magnification and the marker represents 40 μm. Arrows point to apoptotic cells.

Chapter 7

General discussion

Exposure to sunlight results in a rapid reduction in infectivity of baculoviruses. This severely limits their commercial expansion as biocontrol agents for insect pests. UV screening agents need to be added to the virus formulation to extend the field persistence (Black et al., 1997; Moscardi, 1999). The high production costs associated with these chemical UV protectants and the fact that they may affect the virulence of the virus hampers their application (Black et al., 1997). The use of "chemicals" as UV protectants is also incompatible with the use of baculoviruses in biological and organic farming practices. The rapid inactivation of these DNA viruses by sunlight is most likely due to the induction of pyrimidine dimers in the viral DNA by the UV component in the spectrum. Photolyases (PHRs) are enzymes that can repair such dimers in a blue light-dependent manner (Sancar, 2003; Weber, 2005). The baculovirus *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) has two genes (*phr1* and *phr2*) that encode putative class II cyclobutane pyrimidine dimer (CPD) photolyases (van Oers et al., 2005; van Oers et al., 2004). One of these encoded proteins, Cc-PHR2, has been shown to have photoreactivating ability in a heterologous system (bacteria) as well as *in vitro* (van Oers et al., 2008). At the start of this research, only a few other baculoviruses were known to carry *phr* genes (Wang et al., 2008; Willis et al., 2005) and the question is how widespread this property is among baculoviruses and what their mode of action in the virus is.

The biological role of the two ChchNPV photolyases in the replication cycle of the baculovirus is not clear, but the hypothesis is that they provide the baculovirus with an ecological benefit as they repair DNA lesions induced by UV light. The studies described in this thesis focused on: (1) the possible existence of *phr* genes in other baculoviruses, the phylogenetic relationship between baculoviruses and their *phr* genes and the ancestry of these genes, (2) the localization of the two ChchNPV photolyase proteins in insect cells and in occlusion derived virus (ODV) particles, and (3) the biological role of DNA photolyases and the potential for agricultural application by providing UV resistance to a baculoviruses not encoding a PHR protein. In this chapter, the results and data obtained in the previous chapters are discussed in the light of the current state of affairs and further perspectives for the study and application of baculovirus photolyases are given.

Survey of *phr* genes among baculoviruses

According to the complete genomic data available for 53 baculoviruses to date (May 2010), genes encoding putative photolyases were only identified in the genomes of ChchNPV, *Trichoplusia ni* (Tn) SNPV and *Spodoptera litura* (Spli) GV. Based on phylogenetic analysis, both ChchNPV and TnSNPV belong to group II NPVs infecting lepidopteran insects in the subfamily Plusiinae of the Noctuidae (van Oers et al., 2004; Willis et al., 2005). In chapter 2, a PCR-based survey was performed, which showed the presence of photolyase-like genes in five additional group II NPVs infecting plusiine larvae. The identified plusiine baculovirus *phr* genes form three groups which all appear to originate from a common ancestor based on phylogenetic analysis. Two of these groups are formed by *Cc-phr1*

and *Cc-phr2*-like groups. A third group contains the *phr* genes from *Thysanoplusia orichalcea* NPV-B9 and *Pseudoplusia includens* NPV-GT2 (the host of this virus is recently renamed to *Chrysodeixis includens* (Johnson et al., 2010) and is well separated from the two other clades, but still has a clear evolutionary link (Fig. 3a, Chapter 2). This suggests that one or more *phr* genes were introduced in an ancestral plusiine-infecting baculovirus and from there evolved into the *phr* genes that we observe today in members of this group of viruses. ThorNPV-28A-1 on the other hand, which was identified as a group I NPV but also infects a plusiine insect, does not contain a photolyase gene, which is compatible with the view that *phr* genes are typical for plusiine-infecting group II NPVs.

Reconstitution experiments have suggested that Cc-PHR2 uses a 8-HDF as second co-factor (van Oers et al., 2008). However, up to now 8-HDF has been found in four groups of organisms: Archaea, Actinobacteria, Cyanobacteria and unicellular algae (Eker et al., 1989). No information points towards the occurrence of 8-HDF in other organisms like insects, plants or viruses and therefore the source providing the 8-HDF for Cc-PHR2 is not clear. One possible source may be microorganisms that are present in the digestive tract of the host larvae. The presence of members of the class Actinobacteria, which are known to synthesize 8-HDF, in the midgut of gypsy moth larvae has been reported (Broderick et al., 2004). Information on the possible source of the 8-HDF may also provide a clue on the origin of the *phr* genes in Plusiinae-infecting NPVs. Such a situation is similar to the possible origin of chitinases in baculoviruses which may have been derived from the bacterium, *Serratia marcescens*, present in the same ecological niche (Hawtin et al., 1995). Alternatively, Cc-PHR2 does not possess a second co-factor, as recombinant apophotolyase (the photolayse without a co-factor) already exhibits considerable activity *in vivo* (van Oers et al., 2008). This suggests that FAD in Cc-PHR2 might also work as a light-harvesting chromophore as previously reported for *Sulfolobus tokodaii* (Fujihashi et al., 2007).

The phylogeny of *phr* genes also showed that the *phr* gene of SpliGV, which infects *S. litura* in the Xyleninae subfamily of the Noctuidae and which is not an alphabaculovirus but a betabaculovirus (Jehle et al., 2006), is only distantly related to the plusiine NPV photolyases. This suggests that the *phr* from SpliGV has a different origin and evolutionary history and may have been derived from a different source. The two incomplete *phr* ORFs encoded by the group II NPV, which infects *Clanis bilineata* (Family Sphingidae), showed too little overlap with the obtained partial *phr* sequences in the current analysis to allow reliable phylogenetic analysis, but they may be remnants of one photolyase ORF disrupted by mutations or they may in fact be one ORF, which was split as a consequence of sequencing errors.

CPD photolyases are a relatively newly discovered feature for baculoviruses. The question is how this feature was introduced and why it is conserved in group II NPVs that infect plusiine hosts. One possible explanation for the gain of this feature is that an ancestor of these NPVs obtained a copy of a *phr* gene by horizontal gene transfer from its hosts during infection. To test this hypothesis, it is essential to obtain genetic information of the photolyase genes from the plusiine hosts so that the evolutionary relationship between *phrs* from baculoviruses and plusiine insects can be investigated. Insect do have class II DNA photolyase genes as has been found for the lepidopteran insect *Bombyx mori* (Silkworm.genomics.org.cn; Bmb009672) and the dipteran insects *Drosophila melanogaster* (S73530), *D. pseudoobscura* (CM000071) and *Anopheles gambiae* (EAA09350). In order to unravel the *phr* sequences from the plusiine hosts, similar PCR-amplifications can be performed as described in chapter 2 for the viral genes, using the same set of degenerate primers.

Another possible source for these viral *phr's* might be another benefical organism or pathogen associated with the same insect as the viral ancestor of the plusiine group II NPVs, such as another

virus, a bacterium, a fungus or a microsporidium, within the same ancestral host. In Chapter 2, the phylogenetic tree showed that the *phr's* of the entomopoxviruses, *Melanoplus sanguinipes* (MS)EV and *Amsacta moorei* (AM)EV are distinctly related to *phr* genes of the plusiine-infecting NPVs. Recently, it has been shown that the AMEV PHR has photolyase activity in *phr*-negative *E. coli* cells (Nalcacioglu et al., 2010). The resolution of the tree presented in chapter 2 does not allow to conclude whether baculoviruses and (entomo-) poxvirus photolyases originated from the same common ancestor. Further studies are needed for the identification of photolyase genes from the current hosts and other organisms co-existing in or infecting the same hosts to reveal the origin of the baculovirus photolyase genes. In addition, the use of total instead of partial *phr* gene sequences may help to increase the tree resolution in the phylogenetic analysis.

So far, all found baculoviral *phr* genes are observed in single nucleocapsid (S) NPVs or in a GV. Hence, in these type of viruses a single nucleocapsid is packaged per ODV particle and even a single ODV virion is present per GV OB (Miller, 1997b; Rohrmann, 2008a; Slack and Arif, 2006). As a consequence, it is possible that in nature SNPVs and GVs are more vulnerable to UV inactivation than MNPVs, as there is only a single genome copy present per virion. In contrast, MNPVs which have multiple genome copies that end up in a single midgut cell may benefit from complementation or recombination between genomes derived from a single ODV particle. Alternatively, the plusiine host larvae (or their ancestors) may have been exposed more to UV light, due to for instance differences in behaviour than members in other subfamilies of the Noctuidae. They may climb for instance more to the surface of the canopy when infected by a baculovirus.

Localization of Cc-PHRs in insect cells and ChchNPV virions

In a previous study, a complementation assay in an *E. coli* strain that lacks its endogenous DNA repair system showed that Cc-PHR2 is an active CPD photolyase. In addition, oligonucleotides with thymine dimers could be repaired by recombinant Cc-PHR2 protein produced in *E. coli*, which implies a direct interaction of PHRs with DNA (van Oers et al., 2008). Baculoviruses replicate and assemble in the nucleus of insect cells, so the nuclear localization and DNA binding properties are prerequisites for a photolyase to repair (baculovirus) DNA. In chapter 3, the intercellular localization of both photolyase proteins of ChchNPV was studied in *T. ni* High Five cells using a transient expression system and with EGFP as a reporter. The assay demonstrated that the Cc-PHR1-EGFP fusion protein was mainly localized in the nucleus of insect cells. It took more time for Cc-PHR2-EGFP to completely enter the nucleus (Fig. 2&3, Chapter 3). This could be explained by a much higher expression level of *Cc-phr2-egfp* than of *Cc-phr1-egfp*. Immunofluorescence analysis using anti-Cc-PHR2 antibodies showed the quick nuclear localisation of non-fused Cc-PHR2, demonstrating that fusion of Cc-PHR2 to EGFP hindered the nuclear import of the fusion product (Fig. 6, Chapter 3).

In eukaryotic cells, small proteins enter the nucleus via passive diffusion through nuclear pores. Import of proteins larger than approx. 45 kDa is dependent on an energy-dependent transport mechanism to the nucleus. This mechanism involves the recognition of a nuclear localization signal (NLS) present in the protein that needs to be transported by nuclear transport factors (importin) allowing the subsequent translocation through the nuclear pore (Gorlich and Mattaj, 1996; Newmeyer, 1993). Known classical NLSs are generally short stretches of basic amino acids with an abundance of lysine and arginine and are either continuous stretches (monopartite) or two of such amino acid sequences separated by a linker region of 10–12 amino acids (bipartite) (Lange et al., 2007). Up to now, no NLS in any DNA photolyase has been determined.

To predict the NLS of the Cc-PHRs, the specific software PredictNLS was used (http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl), however, no motifs were present in the Cc-PHRs with the same features as known NLS sequences. Therefore, two putative NLS motifs for Cc-PHR2 were selected, that were rich in lysine and arginine and that were present in conserved regions of class II CPD photolyases (RKKLNSKLECYLKPFPP and YLRMYWAKK) assuming that NLS sequences should be conserved in all photolyases (except in those of poxviruses, which replicate in the cytoplasm). Both lysine and arginine residues were substituted by alanine in each or both of the putative NLS sequences. The subcellular localization of transiently expressed Cc-PHR2 with mutation(s) in the putative NLS demonstrated that these two putative NLSs are not crucial for nuclear import of Cc-PHR2 (Fig. 1). Therefore, the mechanism of the nuclear import of Cc-PHRs is currently not understood.
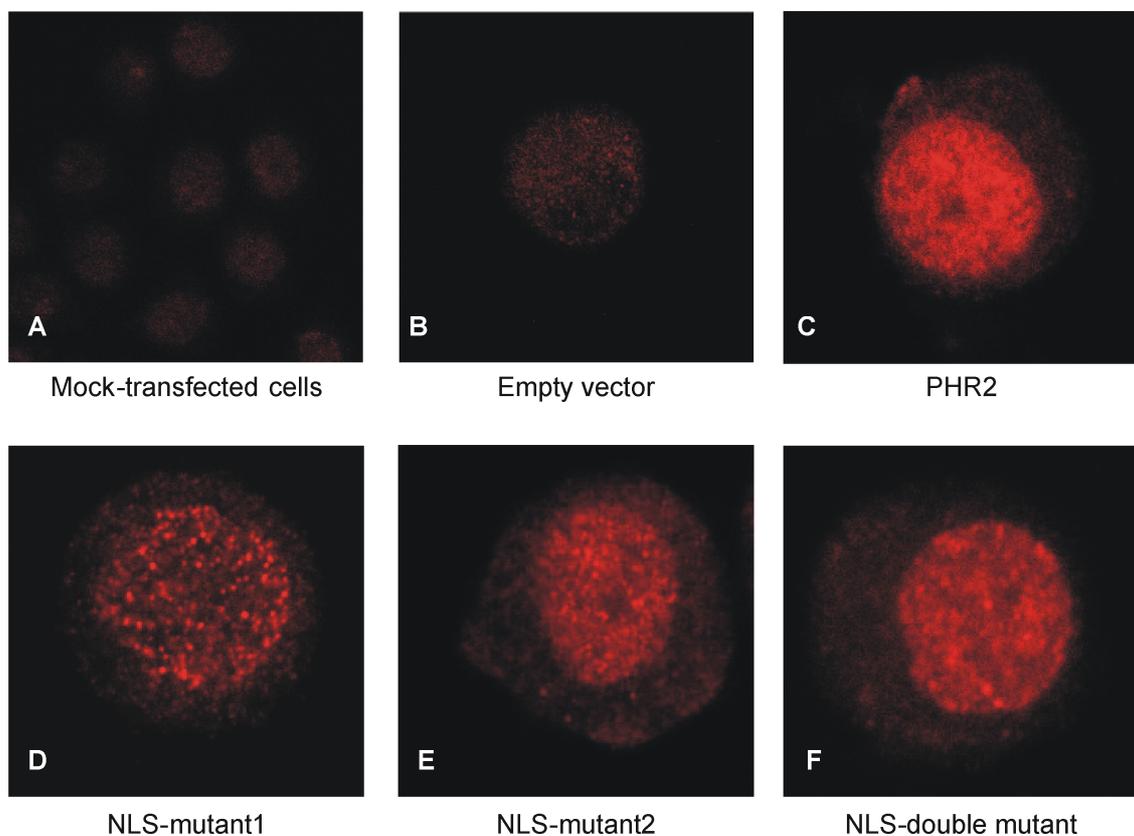
Figure 1. Intracellular localization of PHR2 and NLS-mutant PHR2 proteins in transfected *T. ni* High Five cells by immunofluorescence using anti-Cc-PHR2 serum. No fluorescence was observed in mock-transfected cells (A) and cells transfected with the empty vector (B). Rhodamine fluorescence images are given at 24 h p.t. for the PHR2 expressing cells (C), the cells expressing PHR2-NLS1 mutant (D), the PHR2-NLS2 mutant (E) and PHR2-NLS double mutant (F).

Recently, it was reported for a number of proteins that their nuclear transport does not follow the conventional pathway with a NLS, but that instead they utilize alternative mechanisms, as reviewed by Kylie and David (2009). The newly discovered pathways include facilitation of importin-dependent transport by microtubule motors as well as importin-independent pathways, which involve either other transport molecules such as the calcium-binding protein calmodulin or the direct binding

of the protein to be transported to components of the nuclear pore complex. Interestingly, the cellular cytoskeleton system, for instance the microtubular and actin filament networks, has been shown to play a role in the nuclear import of many viral proteins (Digard et al., 1999; McDonald et al., 2002; Sodeik et al., 1997; Whittaker et al., 2000) as well as of cancer regulatory proteins (Roth et al., 2007). It has also been demonstrated that the cytoskeleton in the host cell is utilized for trafficking of baculovirus nucleocapsids within the infected cells (Kasman and Volkman, 2000; Lanier and Volkman, 1998; Volkman, 2007; Williams and Faulkner, 1997). Cc-PHR-EGFP proteins were found to co-localize with mitotic spindle structures, which are among others comprised of microtubules (chapter 3). Whether the nuclear accumulation of Cc-PHRs is mediated by the cytoskeletal system or by other unknown pathways needs to be explored in the future by the repetition of the transfection assays in the presence of cytoskeletal depolymerizing agents such as cytochalasin D, nocodazole, and colchicine (Christiansen et al., 2006; Wakatsuki et al., 2001). In a later stage cytoskeletal proteins, which interact with PHRs may be identified.

Since the presence of substantial amounts of viral proteins and viral DNA during baculovirus infection could influence the distribution of Cc-PHR proteins in insect cells, the effect of *Autographa californica* (Ac) MNPV infection on the localization of Cc-PHRs was studied. In contrast to the Cc-PHR2-EGFP fusion protein, PHR1-EGFP was found homogeneously in both the cytoplasm and the nucleus in infected cells and was not associated with the virogenic stroma. A previous study indicated that *phr1* does not encode an active photolyase and the alignment of the Cc-PHR1 amino acid sequence with other class II photolyases with known DNA repair activity showed that several strictly conserved amino acids are absent in Cc-PHR1 (van Oers et al., 2008). For instance a conserved tryptophan involved in electron transfer (Aubert et al., 2000) and present in Cc-PHR2 (W355) was not present in Cc-PHR1. Therefore, these different features for Cc-PHR1 suggest that Cc-PHR1 may play another role instead of or in addition to direct DNA repair. The investigation of the existence of photolyase genes in plusiine-infecting group II NPVs (Chapter 2) showed that four viruses besides ChchNPV-NL and TnSNPV have a *Cc-phr1* like gene. In particular, TnSNPV and PsinNPV-GT only have a single *phr1* like gene. In addition, the conserved tryptophans absent in *Cc-phr1* are also not present in these other *phr1* like genes. For none of the *phr1* like genes in these other baculoviruses it is known whether their *phr* genes encode active CPD photolyases. In the case that these PHR1 proteins do not have photorepair activity, the question is what other function they might have during the infection cycle of these plusiine-infecting NPVs. It is unlikely that *phr1* like genes would be retained in the genomes of these baculoviruses when they would not be beneficial for the virus.

It was observed that Cc-PHR fusion proteins are associated with mitotic chromosomes, which consist of substantially condensed DNA (Chapter 3, Fig. 4). Moreover, as described above, PHR2-EGFP colocalized with virogenic stroma in infected cells. Virogenic stroma forms the replication factory where viral DNA is replicated and progeny virions are assembled (Williams and Faulkner, 1997). The collective results obtained provide evidence that Cc-PHRs have the capability of binding to condensed DNA, which is a crucial step to repair the DNA damage within baculovirus nucleocapsids where large amounts of DNA are packaged (Rohrmann, 2008c). However, they may also bind to non-condensed DNA, but this would not have been visible in the experiments performed.

The motifs of ChchNPV PHR proteins and any other class II CPD photolyases involved in DNA binding are not known. The crystal structure of a class I CPD DNA photolyase from *E. coli* demonstrated that the pyrimidine-dinucleotide binding site is located in a cavity, which lies in the centre of the FAD binding domain in close proximity to the FAD molecule (Park et al., 1995; Schulz, 1992). Furthermore, this proposed dimer binding model of *E. coli* photolyase has been confirmed in

yeast (*Saccharomyces cerevisiae*) photolyase by mutational studies (Vande Berg and Sancar, 1998). The overall amino acids similarity between the two classes of CPD photolyases is rather low, which complicates the prediction of the pyrimidine-dinucleotide binding sites of class II photolyases, such as the baculovirus-PHRs. Mutagenesis studies using the transient expression method used in this thesis may be an alternative approach to crystallization studies, especially if combined with synchronizing cells making it easier to observe cells in mitosis. Since Cc-PHR2 has been overexpressed and purified from *E. coli* (van Oers et al., 2004), the three-dimensional structure of Cc-PHR2 could be determined subsequently and once resolved the structure might be used as a model for class II photolyases in future studies.

On the basis of the association with DNA and the fact that the baculovirus PHR proteins could function upon entry in the midgut cells if not before as they only require blue light to repair DNA, one may assume that the PHRs are present in ODVs. Analysing whether Cc-PHRs are present in ChchNPV virions (ODVs and/or BVs) can provide valuable information to understand where and when they perform their function. As photolyase activity only requires blue light, Cc-PHR2 might be able to perform its function outside cells, as has been demonstrated for a poxvirus photolyase (Srinivasan and Tripathy, 2005). PHR2-EGFP was not visible with confocal microscopy in OBs produced in *T. ni* High Five cells upon AcMNPV infection (chapter 3). In addition, PHR2 and PHR2-EGFP were not detected by western blot analysis in OBs isolated from these AcMNPV infected cells either (data not shown). None of the Cc-PHRs was detected in either type of virions by western blotting (data not shown). This may imply there is no PHR protein present or that it is present at a very low level. As reported in literature, there are only 10 to 20 active DNA photolyase molecules in a single *E. coli* cell in the stationary phase (Alcorn and Rupert, 1990). Therefore, a far more sensitive approach, LC-MS/MS was employed to trace Cc-PHRs in ODVs and budded virus (BV) of ChchNPV. However, photolyase peptides were not identified in either ODV (chapter 4). Therefore, DNA repair may occur after infection, when early gene expression has been initiated, as we were not able to show their presence in ChchNPV virions, nor of Cc-PHR2 in recombinant Hear-phr2-polh ODVs (Chapter 5).

Enhanced UV resistance of HearNPV by Cc-PHR2

The previously performed complementation assay in DNA repair deficient *E. coli* cells revealed that Cc-PHR2 has photoreactivation activity and the restoration of thymine dimers by recombinant Cc-PHR2 into monomers was also demonstrated *in vitro* (van Oers et al., 2008). The localization studies in this thesis showed that Cc-PHRs were targeted to the nucleus and interacted with chromosomes indicated that Cc-PHRs could bind DNA. All the obtained data except the absence of PHR2 in ODV point into the direction that Cc-PHR2 may play a role in baculoviral DNA repair. Therefore, the question was whether Cc-PHR2 can protect a baculovirus against UV inactivation. However, ChchNPV could not be used easily for UV resistance bioassays, because it is difficult to generate a Cc-*phr* null virus as a negative control, without the availability of a permissive cell line. With such a cell line conventional recombination and bacmid technology can be applied to generate mutants.

As described above, most baculoviruses do not possess photolyase genes and that is also true for *Helicoverpa armigera* (Hear) NPV (Chen et al., 2001). We choose this virus as it is also a group II NPV and an SNPV, and UV sensitivity studies have shown that this virus is highly sensitive to UV inactivation in the field (Sun et al., 2004a). A recombinant HearNPV harboring *Cc-phr2* was constructed and was used to determine the ability of Cc-PHR2 to enhance the resistance of

baculoviruses against UV irradiation. The UV sensitivity of this recombinant virus was evaluated by comparing the percent mortality caused in *H. armigera* larvae by UV-treated OBs from HearNPV with and without *phr2* gene (chapter 5). The assay showed that the susceptibility to UV was significantly reduced in the HearNPV recombinant with *phr2* as compared to the Hear-NPV *phr2* null virus, but only when OBs were incubated in blue light after UV inactivation, a clear indication that this difference was the consequence of photoreactivation activity. The obtained result showed that Cc-PHR2 can increase the UV resistance of a baculovirus even though a small proportion of the viral genome copies appeared to contain the *Cc-phr2* gene (chapter 5). This means that the effect may be much larger when we can test a pure HearNPV-recombinant virus in the future.

This remarkable finding provided indirect evidence that Cc-PHR2 is probably packaged in ODVs. As discussed in Chapter 5, when photolyases are contained in ODVs, time for transcription and expression of *phr2* after virus entry is not required and mutations in the *phr* gene itself are not detrimental to repair. Cc-PHR2 in ODV can get energy required for the photoreactivation easily as ODVs persist in the environment where visible light is around. Furthermore, it is questionable whether enough light is present in the gut to allow photoreactivation. Hence, a photolyase packaged in ODV might repair viral DNA much more directly and efficiently. To verify this hypothesis, the localization of Cc-PHR2 was investigated in ODV of recombinant HearNPV by western blot assay and LC-MS/MS. However, no Cc-PHR2 was detected in ODVs derived from recombinant Hear-phr2-polh (data not shown), which could be explained by a rather low level of PHR2 proteins in the ODV sample due to the loss of the *phr2* gene in a large fraction of the genomes.

The UV-irradiated HearNPV OBs for the bioassay were treated by photoreactivating light for 6 h to ensure sufficient time to repair all the produced CPDs. Biophysical studies have shown that energy transfer between the two co-factors and to the CPDs followed by splitting of CPDs proceed rapidly, reviewed by (Essen and Klar, 2006). So far, it is not known how long the photoreaction takes in a baculovirus with Cc-PHR2 and when the photorepair starts. However, the time range for the photorepair in baculovirus might be roughly estimated by a time course analysis, for which a similar UV resistance assay (as described in chapter 5) using the blue light treated viruses collected at different time points would be performed by droplet feeding instead of the virus-coated diet. The collected data may determine how long the photoreactivation by Cc-PHR2 takes in a baculovirus.

Wild-type HearNPV was found by a series of PCR analyses in the recombinant HearNPV viral stock derived from a recombinant bacmid. The presence of wild-type viruses was most likely caused by intrinsic instability of the recombinant genomes. Based on the qPCR results, approximately 96% of the genome copies of recombinant HearNPV lost the bacterial cassette. As described in chapter 5, a sequence present in the HaBacHZ8 bacmid (including the HearNPV *polh* promoter and the first 258 and the last 31 nucleotides of the *polh* ORF as well as its downstream poly-A region) was also inserted from the expression cassette to obtain the recombinant HearNPVs genome. These resulted in overlapping regions, which appear to have sufficient length to allow recombination between bacmid molecules, thereby kicking the entire bacterial cassette and transposed insert out and recombining back to wild-type HearNPV (Fig. 4, Chapter 5).

The recombination between two bacmid molecules with the overlapping regions as described above may be one of the explanations for the deletion of *Cc-phr2*. In addition there may be a selective loss of the *Cc-phr2* gene from recombinant HearNPV. This can be seen from the ratio mini-F/*phr2*. A low number (7%) of viruses, in which a bacterial cassette was retained, contained *Cc-phr2*. The overexpressed *Cc-phr2* in HearNPV may be toxic for the cells or may interfere with certain baculoviral activities such as DNA replication and transcription, in which the Cc-PHR2 might

compete for the DNA binding with other enzymes involved in these processes. Interestingly, although the frequency of *Cc-phr2* in the HearNPV genome copies is rather low, the positive results obtained in the bioassay indicate that Cc-PHR2 may provide potent protection of HearNPV from UV inactivation. A very low amount of Cc-PHR2 may be sufficient for the repair of baculovirus DNA. This is in line with the low abundance of CPD photolyase in other systems (Alcorn and Rupert, 1990).

The instability of recombinant HearNPV bacmids is a general problem also observed by others (personal communication) and may be improved by removing the regions in the parental bacmid HaBacHZ8 (Wang et al., 2003), that overlap with the insertion cassette. To this aim, the cre-loxP system with modified loxP sites (Suzuki et al., 2005) was used to delete the polyhedrin promoter and 5' end of the ORF from the HearNPV bacmid (Ros et al. 2010, unpublished data). First, the fragment was replaced by a chloramphenicol resistance (CAT) gene by homologous recombination using the lambda red system (Datsenko and Wanner, 2000). On both sites of the CAT gene, modified loxP sites were introduced (lox66 and lox71). The inserted CAT gene was subsequently removed by expressing cre-recombinase. Cre-recombinase recognizes and fuses both differentially modified, loxP sites, resulting in a single (doubly modified) loxP site (Fig. 2). This doubly modified loxP site is not recognized by cre-recombinase anymore, so that additional mutations to the backbone can be made in the future for instance for functional genomics. The modified region of the resulting bacmid was sequenced, and showed the successful removal of the polyhedrin fragment. The repaired bacmid (HaBacVR1) is currently being tested for infectivity, gene expression and stability (Ros et al., unpublished data). In addition, investigation of the stability of a new HearNPV recombinant carrying *Cc-phr2* derived from the newly generated HaBacVR1 could determine whether the overexpression of *Cc-phr2* induces the loss of this gene. Photorepair activity *in vivo* of Cc-PHR1 could be tested in a similar way as for Cc-PHR2, although it has not been shown to be an active photolyase in *E. coli*. This might provide more information about the potential role of Cc-PHR1 in ChchNPV.
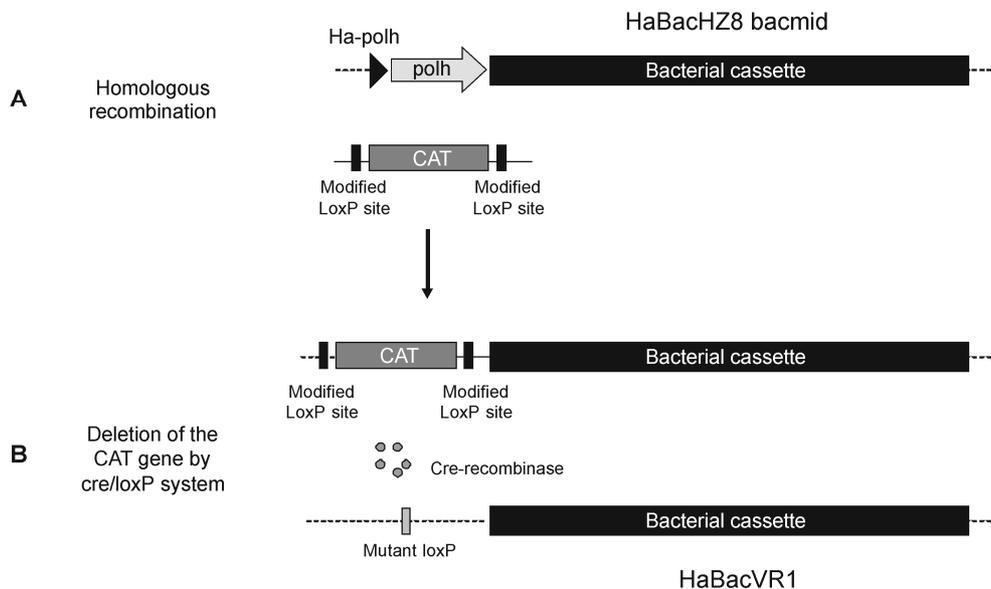
Figure 2. Schematic for deletion of the N-terminal part of the HearNPV polyhedrin sequence remained in the HaBacHZ8 bacmid by the cre/loxP system. The target region is replaced by a chloramphenicol resistance (CAT) gene flanked with modified loxP sites and 50 bp regions homologous to the polyhedrin flanking regions in the bacmid by homologous recombination (A). Cre-recombinase recognizes and fuses both modified loxP sites, thereby excising the CAT gene between the modified loxP sites, leaving a double mutated loxP site, which results in the new HaBacVR1 bacmid (B).

The CcE-1 cell line – a new tool to study Cc-PHRs and plusiine baculoviruses

So far, the functional studies of the Cc-PHRs proteins have been performed *in vitro* or in heterologous systems. However, whether the CPD photolyase genes play a role in protecting ChchNPV against UV inactivation has not been shown yet. To approach this issue, a Cc-*phr*-deleted or knock-out ChchNPV is essential. In order to study the CPD photolyase genes of ChchNPV in detail and to better understand the ChchNPV pathology and biological functions of for instance the unique ChchNPV proteins, a cell line, named WU-CcE-1, was established (Chapter 6) from embryos of the insect *C. chalcites*. The CcE-1 cells were permissive for infection by ChchNPV and OBs produced in these cells were infectious for *C. chalcites* larvae. CcE-1 cells were also permissive for the related TnSNPV. The evolutionary relationship between ChchNPV and TNSNPV is close (Herniou and Jehle, 2007; Xu et al., 2008) and the two viral genomes are highly collinear (van Oers et al., 2005; Willis et al, 2005). This suggests that they may have a (partial) overlapping host range and might explain why CcE-1 cells are susceptible to both baculoviruses. *T. ni* High Five cells on the other hand are only semi permissive for ChchNPV (van Oers et al., 2004).

This newly established CcE-1 cell line is a useful tool for the further study of ChchNPV photolyases. In a previous study, *Cc-phr2* was predicted to be an early gene product based on the presence of consensus GATA motifs, which is a host derived promoter motif (van Oers et al., 2004). It has been shown that this gene is indeed expressed in infected *T. ni* cells and *C. chalcites* larvae before (van Oers et al., 2004). The transcription of *Cc-phr1* has not been analyzed so far. Based on the genome data of ChchNPV, a CATG motif characteristic for early baculovirus transcription initiation is present at position -17 upstream from the *phr1* ATG start codon, preceded by a TATA motif at -45. Whether *Cc-phr1* has a transcriptional ability and whether it can be expressed in ChchNPV infected cells should be determined using CcE-1 cells. The transcription profile of *Cc-phr1* and *Cc-phr2* should be determined in infected CcE-1 cells, in the presence or absence of inhibitors of protein or DNA synthesis, allowing the discrimination between immediate-early, delayed early and late genes. To allow packaging of Cc-PHRs into ODVs, which we could not show yet, expression during the late phase of infection, when ODVs are assembled, is likely to be required.

Concluding remarks and future perspectives

In this PhD thesis the presence of CPD photolyase genes in baculoviruses infecting plusiinae insect larvae was demonstrated, ChchNPV PHR proteins localized in the nucleus, and more specifically PHR2 associated with virogenic stroma in infected cells where virial DNA replication occurs. This location is compatible with their function as DNA photolyase. A new permissive cell line was developed for future studies of Cc-PHRs and ChchNPV. More information has been obtained for *Cc-phr2* than for *Cc-phr1* and the information collected so far indicated that *Cc-phr1* may have a different function than *Cc-phr2*. *Cc-phr2* had been shown to have photorepair ability in *E. coli* and *in vitro*. In this thesis it was shown that it improved the UV resistance of HearNPV and therefore it has potential as UV protectant in commercial biocontrol agents based on baculoviruses. The UV resistance assay with Hear-phr2-polh provided a clue that Cc-PHR2 might be packaged in ODVs. However, the inability to find Cc-PHR2 in ChchNPV and recombinant HearNPV ODVs complicates our understanding of the mechanism of photorepair by Cc-PHR2. Where, when and how Cc-PHR2 performs DNA repair in ChchNPV remains enigmatic at this moment.

Further studies are needed using the newly developed HearNPV bacmid to analyze the affect of Cc-PHR2 on UV resistance both in the laboratory and in the field in detail, in order to determine the persistence of recombinant HearNPV containing *Cc-phr2* in the field and to analyze its feasibility for the biological control of the cotton bollworm. If protective, the incorporation of Cc-*phr2* may be expanded to other baculovirus based biocontrol agents, *e.g. Cydia pomonella* GV, used in Europe to protect apple and pear. In this study, two other *Cc-phr2* like genes were discovered in Plusiinae-infecting group II NPVs, and it would be interesting to see whether these gene also encode active enzymes.

The DNA repair activity of Cc-PHR2 may also be tested in mammalian cells since placental mammals do not possess photoreactivation enzymes to repair DNA damage, but instead use the NER repair system, which is less efficient than photoreactivation. If Cc-PHR2 is active in mammalian or human cells, especially those isolated from patients with a deficiency in the NER system (*e.g.* xeroderma pigmentosa patients), it might be a potential candidate to reduce the risk for UV induced skin diseases, for instance in combination with the baculovirus mediated gene delivery system.

References

Aebersold, R., and Mann, M., 2003. Mass spectrometry-based proteomics. Nature 422, 198-207.

Afonso, C.L., Tulman, E.R., Lu, Z., Oma, E., Kutish, G.F., and Rock, D.L., 1999. The genome of *Melanoplus sanguinipes* entomopoxvirus. J. Virol. 73, 533-552.

Afonso, C.L., Tulman, E.R., Lu, Z., Zsak, L., Kutish, G.F., and Rock, D.L., 2000. The genome of fowlpox virus. J. Virol. 74, 3815-3831.

Akasaka, S., and Yamamoto, K., 1991. Construction of *Escherichia coli* K12 *phr* deletion and insertion mutants by gene replacement. Mutat. Res. 254, 27-35.

Alcorn, J.L., and Rupert, C.S., 1990. Regulation of photolyase in *Escherichia coli* K-12 during adenine deprivation. J. Bacteriol. 172, 6885-6891.

Aubert, C., Vos, M.H., Mathis, P., Eker, A.P.M., and Brettel, K., 2000. Intraprotein radical transfer during photoactivation of DNA photolyase. Nature 405, 586-570.

Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M., and Possee, R.D., 1994. The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202, 586-605.

Bawden, A.L., Glassberg, K.J., Diggans, J., Shaw, R., Farmerie, W., and Moyer, R.W., 2000. Complete genomic sequence of the *Amsacta moorei* entomopoxvirus: Analysis and comparison with other poxviruses. Virology 274, 120-139.

Beljelarskaya, S.N., 2002. A baculovirus expression system for insect cells. Mol. Biol. 36, 281-292.

Bennett, C.J., Webb, M., Willer, D.O., and Evans, D.H., 2003. Genetic and phylogenetic characterization of the type II cyclobutane pyrimidine dimer photolyases encoded by Leporipoxviruses. Virology 315, 10-19.

Bideshi, D.K., Anwar, A.T., and Federici, B.A., 1999. A baculovirus anti-apoptosis gene homolog of the *Trichoplusia ni* granulovirus. Virus Genes 19, 95-101.

Black, B.C., Brennan, L.A., and Dierks, P.M., 1997. Commercialization of baculoviral insecticides. In: L.K. Miller (Ed), The Baculoviruses, pp. 341-387. Plenum Press, New York, USA.

Blissard, G.W., 1996. Baculovirus-insect cell interactions. Cytotechnology 20, 73-93.

Blissard, G.W., and Rohrmann, G.F., 1990. Baculovirus diversity and molecular biology. Annu. Rev. Entomol. 35, 127-155.

Braunagel, S.C., Elton, D.M., Ma, H., and Summers, M.D., 1996a. Identification and analysis of an *Autographa californica* nuclear polyhedrosis virus structural protein of the occlusion-derived virus envelope: ODV-E56. Virology 217, 97-110.

Braunagel, S.C., He, H., Ramamurthy, P., and Summers, M.D., 1996b. Transcription, translation, and cellular localization of three *Autographa californica* nuclear polyhedrosis virus structural proteins: ODV-E18, ODV-E35, and ODV-EC27. Virology 222, 100-114.

Braunagel, S.C., Burks, J.K., Rosas-Acosta, G., Harrison, R.L., Ma, H., and Summers, M.D., 1999. Mutations within the *Autographa californica* nucleopolyhedrovirus *FP25K* gene decrease the accumulation of ODV-E66 and alter its intranuclear transport. J. Virol. 73, 8559-8570.

Braunagel, S.C., Guidry, P.A., Rosas-Acosta, G., Engelking, L., and Summers, M.D., 2001. Identification of BV/ODV-C42, an *Autographa californica* nucleopolyhedrovirus *orf101*-encoded structural protein detected in infected-cell complexes with ODV-EC27 and p78/83. J. Virol. 75, 12331-12338.

References

Braunagel, S.C., Russell, W.K., Rosas-Acosta, G., Russell, D.H., and Summers, M.D., 2003. Determination of the protein composition of the occlusion-derived virus of *Autographa californica* nucleopolyhedrovirus. Proc. Natl. Acad. Sci. USA. 100, 9797-9802.

Broderick, N.A., Raffa, K.F., Goodman, R.M., and Handelsman, J., 2004. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Appl. Environ. Microbiol. 70, 293-300.

Cameron, C., Hota-Mitchell, S., Chen, L., Barrett, J., Cao, J.X., Macaulay, C., Willer, D., Evans, D., and McFadden, G., 1999. The complete DNA sequence of myxoma virus. Virology 264, 298-318.

Chen, W., Li, Z., Li, S., Li, L., Yang, K., and Pang, Y., 2006. Identification of *Spodoptera litura* multicapsid nucleopolyhedrovirus ORF97, a novel protein associated with envelope of occlusion-derived virus. Virus Genes 32, 79-84.

Chen, X., Li, M., Sun, X., Arif, B.M., Hu, Z., and Vlak, J.M., 2000a. Genomic organization of *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus. Arch. Virol. 145, 2539-2555.

Chen, X., Sun, X., Hu, Z., Li, M., O'-Reilly, D.R., Zuidema, D., and Vlak, J.M., 2000b. Genetic engineering of *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus as an improved pesticide. J. Invertebr. Pathol. 76, 140-146.

Chen, X., Ukel, W.F.J., Tarchini, R., Sun, X., Sandbrink, H., Wang, H., Peters, S., Zuidema, D., Lankhorst, R.K., Vlak, J.M., and Hu, Z., 2001. The sequence of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus genome. J. Gen. Virol. 82, 241-257.

Cheng, X.-W., Carner, G.R., Lange, M., Jehle, J.A., and Arif, B.M., 2005. Biological and molecular characterization of a multicapsid nucleopolyhedrovirus from *Thysanoplusia orichalcea* (L.) (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 88, 126-135.

Chigançás, V., Batista, L.F.Z., Brumatti, G., Amarante-Mendes, G.P., Yasui, A., and Menck, C.F.M., 2002. Photorepair of RNA polymerase arrest and apoptosis after ultraviolet irradiation in normal and XPB deficient rodent cells. Cell Death Differ. 9, 1099-1107.

Christiansen, J.J., Weimbs, T., Bander, N., and Rajasekaran, A.K., 2006. Differing effects of microtubule depolymerizing and stabilizing chemotherapeutic agents on t-SNARE-mediated apical targeting of prostate-specific membrane antigen. Mol. Cancer Ther. 5, 2468-2473.

Clem, R., 2005. The role of apoptosis in defense against baculovirus infection in insects. In: D.E. Griffin (Ed), Role of Apoptosis in Infection, pp. 113-129. Springer Berlin Heidelberg.

Clem, R.J., 2007. Baculoviruses and apoptosis: a diversity of genes and responses. Curr. Drug Targets 8, 1069-1074.

Clem, R.J., Fechheimer, M., and Miller, L.K., 1991. Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science 254, 1388-1390.

Cohen, D., Marek, M., Davies, B., Vlak, J., and van Oers, M., 2009. Encyclopedia of *Autographa californica* nucleopolyhedrovirus genes. Virol. Sin. 24, 359-414.

Cooper, D., Cory, J.S., Theilmann, D.A., and Myers, J.H., 2003. Nucleopolyhedroviruses of forest and western tent caterpillars: cross-infectivity and evidence for activation of latent virus in high-density field populations. Ecol. Entomol. 28, 41-50.

Cory, J.S., Clarke, E.E., Brown, M.L., Hails, R.S., and O'-Reilly, D.R., 2004. Microparasite manipulation of an insect: the influence of the *egt* gene on the interaction between a baculovirus and its lepidopteran host. Funct. Ecol. 18, 443-450.

Cox, J., and Mann, M., 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367-1372.

Cox, M.M.J., and Hollister, J.R., 2009. FluBlok, a next generation influenza vaccine manufactured in insect cells. Biologicals 37, 182-189.

Dalhus, B., Laerdahl, J.K., Backe, P.H., and Bjørås, M., 2009. DNA base repair - Recognition and initiation of catalysis. FEMS Microbiol. Rev. 33, 1044-1078.

Datsenko, K.A., and Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97, 6640-6645.

Deisenhofer, J., 2000. DNA photolyases and cryptochromes. Mutat. Res. DNA Repair 460, 143-149.

Deng, F., Wang, R., Fang, M., Jiang, Y., Xu, X., Wang, H., Chen, X., Arif, B.M., Guo, L., Wang, H., and Hu, Z., 2007. Proteomics analysis of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus identified two new occlusion-derived virus-associated proteins, HA44 and HA100. J. Virol. 81, 9377-9385.

Depner, K.R., Bouma, A., Koenen, F., Klinkenberg, D., Lange, E., de Smit, H., and Vanderhallen, H., 2001. Classical swine fever (CSF) marker vaccine: Trial II. Challenge study in pregnant sows. Vet. Microbiol. 83, 107-120.

Digard, P., Elton, D., Bishop, K., Medcalf, E., Weeds, A., and Pope, B., 1999. Modulation of nuclear localization of the influenza virus nucleoprotein through interaction with actin filaments. J. Virol. 73, 2222-2231.

Domon, B., and Aebersold, R., 2006. Mass spectrometry and protein analysis. Science 312, 212-217.

Eker, A.P.M., Hessels, J.K.C., and Meerwaldt, R., 1989. Characterization of an 8-hydroxy-5-deazaflavin: NADPH oxidoreductase from *Streptomyces griseus*. BBA-Gen Subjects 990, 80-86.

Eker, A.P.M., Quayle, C., Chaves, I., and van der Horst, G.T.J., 2009. Direct DNA damage reversal: Elegant solutions for nasty problems. Cell. Mol. Life Sci. 66, 968-980.

Essen, L.O., 2006. Photolyases and cryptochromes: Common mechanisms of DNA repair and light-driven signaling? Curr. Opin. Struct. Biol. 16, 51-59.

Essen, L.O., and Klar, T., 2006. Light-driven DNA repair by photolyases. Cell. Mol. Life Sci. 63, 1266-1277.

Fang, M., Wang, H., Wang, H., Yuan, L., Chen, X., Vlak, J.M., and Hu, Z., 2003. Open reading frame 94 of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus encodes a novel conserved occlusion-derived virion protein, ODV-EC43. J. Gen. Virol. 84, 3021-3027.

Faulkner, P., Kuzio, J., Williams, G.V., and Wilson, J.A., 1997. Analysis of P74, a PDV envelope protein of *Autographa californica* nucleopolyhedrovirus required for occlusion body infectivity *in vivo*. J. Gen. Virol. 78, 3091-3100.

Federici, B.A., 1997. Baculovirus pathogenesis. In: L.K. Miller (Ed), The Baculoviruses, pp. 33-59. Plenum Press, New York, USA.

Fitt, G.P., 1989. The ecology of *Heliothis* species in relation to agroecosystems. Annu. Rev. Entomol. 34, 17-52.

Friedberg, E.C., 2003. DNA damage and repair. Nature 421, 436-440.

Friedberg, E.C., Walker, G.C., and Siede, W., 1995. DNA Repair and Mutagenesis, 24-31 pp. ASM Press, Washington, D.C.

Fritsch, E., and Huber, J., 1985. Inactivation of codling moth granulosis viruses by ultraviolet radiation and temperature. Nachr. Dtsch. Pflanzenschutzd. 37, 84-88.

References

Fujihashi, M., Numoto, N., Kobayashi, Y., Mizushima, A., Tsujimura, M., Nakamura, A., Kawarabayasi, Y., and Miki, K., 2007. Crystal structure of archaeal photolyase from *Sulfolobus tokodaii* with two FAD Molecules: Implication of a novel light-harvesting cofactor. J. Mol. Biol. 365, 903-910.

Gao, S.-Y., 1958. Culturing all types of silkworm tissues using the monolayer culture. Chin. Sci. Bull. 7, 219–220

Gombart, A.F., Pearson, M.N., Rohrmann, G.F., and Beaudreaau, G.S., 1989. A baculovirus polyhedral envelope-associated protein: genetic location, nucleotide sequence, and immunocytochemical characterization. Virology 169, 182-193.

Gorlich, D., and Mattaj, I.W., 1996. Nucleocytoplasmic transport. Science 271, 1513-1519.

Grace, T.D.C., 1962. Establishment of four strains of cells from insect tissues grown *in vitro*. Nature 195, 788-789.

Granados, R.R., and Lawler, K.A., 1981. *In vivo* pathway of *Autographa californica* baculovirus invasion and infection. Virology 108, 297-308.

Granados, R.R., Guoxun, L., Derksen, A.C.G., and McKenna, K.A., 1994. A new insect cell line from *Trichoplusia ni* (BTI-Tn-5B1-4) susceptible to *Trichoplusia ni* single enveloped nuclear polyhedrosis virus. J. Invertebr. Pathol. 64, 260-266.

Gross, C.H., Wolgamot, G.M., Russell, R.L.Q., Pearson, M.N., and Rohrmann, G.F., 1993. A baculovirus encoded 16-kDa glycoprotein localizes near the nuclear membrane of infected cells. Virology 192, 386-390.

Harper, D.M., 2009. Current prophylactic HPV vaccines and gynecologic premalignancies. Curr. Opin. Obstet. Gynecol. 21, 457-464

Harrison, R.L., and Summers, M.D., 1995. Mutations in the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus 25 kDa protein gene result in reduced virion occlusion, altered intranuclear envelopment and enhanced virus production. J. Gen. Virol. 76, 1451-1459.

Haseltine, W.A., Gordon, L.K., Lindan, C.P., Grafstrom, R.H., Shaper, N.L., and Grossman, L., 1980. Cleavage of pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA-glycosylase of *M. luteus*. Nature 285, 634-641.

Hawtin, R.E., Arnold, K., Ayres, M.D., de A. Zanotto, P.M., Howard, S.C., Gooday, G.W., Chappell, L.H., Kitts, P.A., King, L.A., and Possee, R.D., 1995. Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. Virology 212, 673-685.

Hearst, J.E., 1995. The structure of photolyase: Using photon energy for DNA repair. Science 268, 1858-1859.

Herniou, E.A., and Jehle, J.A., 2007. Baculovirus phylogeny and evolution. Curr. Drug Targets 8, 1043-1050.

Hershberger, P.A., Dickson, J.A., and Friesen, P.D., 1992. Site-specific mutagenesis of the 35-kilodalton protein gene encoded by *Autographa californica* nuclear polyhedrosis virus: cell line-specific effects on virus replication. J. Virol. 66, 5525-5533.

Hitchman, R.B., Possee, R.D., and King, L.A., 2009. Baculovirus expression systems for recombinant protein production in insect cells. Recent Pat. Biotechnol. 3, 46-54.

Hu, Y., 2006. Baculovirus vectors for gene therapy. Adv. Virus Res. 68, 287-320.

Hu, Y.C., 2005. Baculovirus as a highly efficient expression vector in insect and mammalian cells. Acta Pharmacol. Sin. 26, 405-416.

Hu, Y.C., 2008. Baculoviral vectors for gene delivery: A review. Curr. Gene Ther. 8, 54-65.

Hughes, D.S., Possee, R.D., and King, L.A., 1993. Activation and detection of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects. Virology 194, 608-615.

Hughes, P.R., and Wood, H.A., 1981. A synchronous peroral technique for the bioassay of insect viruses. J. Invertebr. Pathol. 37, 154-159.

Ignoffo, C.M., and Garcia, C., 1992. Combinations of environmental factors and simulated sunlight affecting activity of inclusion bodies of the *Heliothis* (Lepidoptera: Noctuidae) nucleopolyhedrosis virus. Environ. Entomol. 21, 210-213.

IJkel, W.F.J., van Strien, E.A., Heldens, J.G.M., Broer, R., Zuidema, D., Goldbach, R.W., and Vlak, J.M., 1999. Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. J. Gen. Virol. 80, 3289-3304.

Inceoglu, A.B., Kamita, S.G., and Hammock, B.D., 2006. Genetically modified baculoviruses: a historical overview and future outlook. Adv. Virus Res. 68, 323-360.

Jaques, R.P., 1985. Stability of insect viruses in the environment. In: K. Maramorosch and K.E. Sherman (Eds), Viral Insecticides for Biological Control, pp. 285-360. Academic Press, San Diego.

Jarvis, D.L., Richard, R.B., and Deutscher, M.P., 2009. Chapter 14 Baculovirus-insect cell expression systems, Methods Enzymol., 463, 191-222.

Jehle, J.A., Blissard, G.W., Bonning, B.C., Cory, J.S., Herniou, E.A., Rohrmann, G.F., Theilmann, D.A., Thiem, S.M., and Vlak, J.M., 2006a. On the classification and nomenclature of baculoviruses: A proposal for revision. Arch. Virol. 151, 1257-1266.

Jehle, J.A., Lange, M., Wang, H., Hu, Z., Wang, Y., and Hauschild, R., 2006b. Molecular identification and phylogenetic analysis of baculoviruses from Lepidoptera. Virology 346, 180-193.

Johnson, J.A., Bitra, K., Zhang, S., Wang, L., Lynn, D.E., and Strand, M.R., 2010. The UGA-CiE1 cell line from *Chrysodeixis includens* exhibits characteristics of granulocytes and is permissive to infection by two viruses. Insect Biochem. Mol. Biol. 40, 394-404.

Jones, K.A., Moawad, G., McKinley, D.J., and Grzywacz, D., 1993. The effect of natural sunlight on *Spodoptera littoralis* nuclear polyhedrosis virus. Biocontrol Sci. Technol. 3, 189-197.

Kamita, S.G., Nagasaka, K., Chua, J.W., Shimada, T., Mita, K., Kobayashi, M., Maeda, S., and Hammock, B.D., 2005. A baculovirus-encoded protein tyrosine phosphatase gene induces enhanced locomotory activity in a lepidopteran host. Proc. Natl. Acad. Sci. USA 102, 2584-2589.

Kasman, L.M., and Volkman, L.E., 2000. Filamentous actin is required for lepidopteran nucleopolyhedrovirus progeny production. J. Gen. Virol. 81, 1881-1888.

Ke, J., Wang, J., Deng, R., and Wang, X., 2008. *Autographa californica* multiple nucleopolyhedrovirus *ac66* is required for the efficient egress of nucleocapsids from the nucleus, general synthesis of preoccluded virions and occlusion body formation. Virology 374, 421-431.

Kikhno, I., Gutierrez, S., Croizier, L., Croizier, G., and Ferber, M.L., 2002. Characterization of *pif*, a gene required for the *per os* infectivity of *Spodoptera littoralis* nucleopolyhedrovirus. J. Gen. Virol. 83, 3013-3022.

Kimura, S., Tahira, Y., Ishibashi, T., Mori, Y., Mori, T., Hashimoto, J., and Sakaguchi, K., 2004. DNA repair in higher plants; photoreactivation is the major DNA repair pathway in non-

References

proliferating cells while excision repair (nucleotide excision repair and base excision repair) is active in proliferating cells. Nucl. Acids Res. 32, 2760-2767.

Kirschbaum, J.B., 1985. Potential implication of genetic engineering and other biotechnologies to insect control. Annu. Rev. Entomol. 30, 51-70.

Kogan, P.H., and Blissard, G.W., 1994. A baculovirus *gp64* early promoter is activated by host transcription factor binding to CACGTG and GATA elements. J. Virol. 68, 813-822.

Komori, H., Masui, R., Kuramitsu, S., Yokoyama, S., Shibata, T., Inoue, Y., and Miki, K., 2001. Crystal structure of thermostable DNA photolyase: Pyrimidine-dimer recognition mechanism. Proc. Natl. Acad. Sci. USA 98, 13560-13565.

Kool, M., Ahrens, C.H., Goldbach, R.W., Rohrmann, G.F., and Vlak, J.M., 1994a. Identification of genes involved in DNA replication of the *Autographa californica* baculovirus. Proc. Natl. Acad. Sci. USA. 91, 11212-11216.

Kool, M., Ahrens, C.H., Vlak, J.M., and Rohrmann, G.F., 1995. Replication of baculovirus DNA. J. Gen. Virol. 76, 2103-2118.

Kool, M., Voeten, J.T.M., Goldbach, R.W., and Vlak, J.M., 1994b. Functional mapping of regions of the *Autographa californica* nuclear polyhedrosis viral genome required for DNA replication. Virology 198, 680-689.

Kost, T.A., and Condreay, J.P., 2002. Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol. 20, 173-180.

Kundu, L.M., Linne, U., Marahiel, M., and Carell, T., 2004. RNA is more UV resistant than DNA: The formation of UV-induced DNA lesions is strongly sequence and conformation dependent. Chem. Eur. J. 10, 5697-5705.

Kylie, M.W., and David, A.J., 2009. Importins and beyond: non-conventional nuclear transport mechanisms. Traffic 10, 1188-1198.

Lacey, L.A., Thomson, D., Vincent, C., and Arthurs, S.P., 2008. Codling moth granulovirus: A comprehensive review. Biocontrol Sci. Technol. 18, 639-663.

Lange, A., Mills, R.E., Lange, C.J., Stewart, M., Devine, S.E., and Corbett, A.H., 2007. Classical nuclear localization signals: definition, function, and interaction with importin α. J. Biol. Chem. 282, 5101-5105.

Lanier, L.M., and Volkman, L.E., 1998. Actin binding and nucleation by *Autographa california* M nucleopolyhedrovirus. Virology 243, 167-177.

Lapointe, R., Popham, H.J.R., Straschil, U., Goulding, D., O'-Reilly, D.R., and Olszewski, J.A., 2004. Characterization of two *Autographa californica* nucleopolyhedrovirus proteins, Ac145 and Ac150, which affect oral infectivity in a host-dependent manner. J. Virol. 78, 6439-6448.

Lehmann, A.R., 1995. Nucleotide excision repair and the link with transcription. Trends Biochem. Sci. 20, 402-405.

Lesch, H.P., Turpeinen, S., Niskanen, E.A., Mahonen, A.J., Airenne, K.J., and Yla-Herttuala, S., 2008. Generation of lentivirus vectors using recombinant baculoviruses. Gene Ther. 15, 1280-1286.

Li, J., Uchida, T., Todo, T., and Kitagawa, T., 2006. Similarities and differences between cyclobutane pyrimidine dimer photolyase and (6-4) photolyase as revealed by resonance Raman spectroscopy. J. Biol. Chem. 281, 25551-25559.

Li, Q., Donly, C., Li, L., Willis, L.G., Theilmann, D.A., and Erlandson, M., 2002. Sequence and organization of the *Mamestra configurata* nucleopolyhedrovirus genome. Virology 294, 106-121.

Lima-Bessa, K.M., and Menck, C.F.M., 2005. Skin cancer: Lights on genome lesions. Curr. Biol. 15.

Ljungman, M., and Zhang, F., 1996. Blockage of RNA polymerase as a possible trigger for UV light-induced apoptosis. Oncogene 13, 823-831.

Lu, A., and Carstens, E.B., 1992. Nucleotide sequence and transcriptional analysis of the *p80* gene of *Autographa californica* nuclear polyhedrosis virus: A homologue of the *Orgyia pseudotsugata* nuclear polyhedrosis virus capsid-associated gene. Virology 190, 201-209.

Luckow, V.A., Lee, S.C., Barry, G.F., and Olins, P.O., 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J. Virol. 67, 4566-4579.

Luque, T., Finch, R., Crook, N., O'Reilly, D.R., and Winstanley, D., 2001. The complete sequence of the *Cydia pomonella* granulovirus genome. J. Gen. Virol. 82, 2531-2547.

Lynn, D.E., 1996. Development and characterization of insect cell lines. Cytotechnol. 20, 3-11.

Lynn, D.E., 1999. Development of insect cell lines: virus susceptibility and applicability to prawn cell culture. Methods Cell Sci. 21, 173-181.

Lytle, C.D., and Sagripanti, J.-L., 2005. Predicted inactivation of viruses of relevance to biodefense by solar radiation. J. Virol. 79, 14244-14252.

Maxwell, K.L., and Frappier, L., 2007. Viral Proteomics. Microbiol. Mol. Biol. Rev. 71, 398-411.

McCarthy, C.B., and Theilmann, D.A., 2008. AcMNPV *ac143 (odv-e18)* is essential for mediating budded virus production and is the 30th baculovirus core gene. Virology 375, 277-291.

McCutchen, B.F., Choudary, P.V., Crenshaw, R., Maddox, D., Kamita, S.G., Palekar, N., Volrath, S., Fowler, E., Hammock, B.D., and Maeda, S., 1991. Development of a recombinant baculovirus expressing an insect-selective neurotoxin: potential for pest control. Bio/Technology 9, 848-852.

McDonald, D., Vodicka, M.A., Lucero, G., Svitkina, T.M., Borisy, G.G., Emerman, M., and Hope, T.J., 2002. Visualization of the intracellular behavior of HIV in living cells. J. Cell Biol. 159, 441-452.

McIntosh, A.H., and Ignoffo, C.M., 1981. Replication and infectivity of the single-embedded nuclear polyhedrosis virus, *Baculovirus heliothis*, in homologous cell lines. J. Invertebr. Pathol. 37, 258-264.

McIntosh, A.H., Grasela, J.J., and Matted, R.L., 1996. Identification of insect cell lines by DNA amplification fingerprinting (DAF). Insect Mol. Biol. 5, 187-195.

Miller, L.K., 1995. Genetically engineered insect virus pesticides: present and future. J. Invertebr. Pathol. 65, 211-216.

Miller, L.K., 1997a. Auxiliary genes of baculoviruses, The Baculoviruses, pp. 267–300 Plenum Press, New York, USA.

Miller, L.K., 1997b. The Baculoviruses. Plenum Press, New York, USA.

Mitchell, D.L., and Nairn, R.S., 1989. The biology of the (6-4) photoproduct. Photochem. Photobiol. 49, 805-819.

Moscardi, F., 1989. Use of viruses for pest control in Brazil: the case of the nuclear polyhedrosis virus of the soybean caterpillar, anticarsia gemmatalis. Mem. Inst. Oswaldo Cruz 84, 51-56.

Moscardi, F., 1999. Assessment of the application of baculoviruses for control of Lepidoptera. Annu. Rev. Entomol. 44, 257-289.

Moser, B.A., Becnel, J.J., White, S.E., Afonso, C., Kutish, G., Savita, S., and Almira, E., 2001. Morphological and molecular evidence that *Culex nigripalpus* baculovirus is an unusual member of the family *Baculoviridae*. J. Gen. Virol. 82, 283-297.

References

Müller, M., and Carell, T., 2009. Structural biology of DNA photolyases and cryptochromes. Curr. Opin. Struct. Biol. 19, 277-285.

Müller, R., Pearson, M.N., Russell, R.L.Q., and Rohrmann, G.F., 1990. A capsid-associated protein of the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata*: genetic location, sequence, transcriptional mapping, and immunocytochemical characterization. Virology 176, 133-144.

Murphy, T.M., and Gordon, M.P., 1981. Photobiology of RNA viruses. In: H. Fraenkel-Conrat and R.R. Wagner (Eds), Comprehensive virology, pp. 285-351. Plenum Press, New York, USA.

Nalcacioglu, R., Akturk, Y., Vlak, J.M., Demirbag, Z., and van Oers, M.M., 2010. *Amsacta moorei* entomopoxvirus encodes a functional DNA photolyase (AMV025) (submitted).

Newmeyer, D.D., 1993. The nuclear pore complex and nucleocytoplasmic transport. Curr. Opin. Cell Biol. 5, 395-407.

Nishigaki, R., Mitani, H., and Shima, A., 1998. Evasion of UVC-induced apoptosis by photorepair of cyclobutane pyrimidine dimers. Exp. Cell Res. 244, 43-53.

Noad, R., and Roy, P., 2003. Virus-like particles as immunogens. Trends Microbiol. 11, 438-444.

O'Connor, K.A., McBride, M.J., West, M., Yu, H., Trinh, L., Yuan, K., Lee, T., and Zusman, D.R., 1996. Photolyase of *Myxococcus xanthus*, a Gram-negative eubacterium, is more similar to photolyases found in archaea and "higher" eukaryotes than to photolyases of other eubacteria. J. Biol. Chem. 271, 6252-6259.

O'Reilly, D.R., and Miller, L.K., 1991. Improvement of a baculovirus pesticide by deletion of the *egt* gene. Nat. Biotechnol. 9, 1086-1089.

Ohkawa, T., Washburn, J.O., Sitapara, R., Sid, E., and Volkman, L.E., 2005. Specific binding of *Autographa californica* M nucleopolyhedrovirus occlusion-derived virus to midgut cells of *Heliothis virescens* larvae is mediated by products of pif genes Ac119 and Ac022 but not by Ac115. J. Virol. 79, 15258-15264.

Olszewski, J., and Miller, L.K., 1997a. Identification and characterization of a baculovirus structural protein, VP1054, required for nucleocapsid formation. J. Virol. 71, 5040-5050.

Olszewski, J., and Miller, L.K., 1997b. A role for baculovirus GP41 in budded virus production. Virology 233, 292-301.

Öztürk, N., Kao, Y.T., Selby, C.P., Kavakli, I.H., Partch, C.L., Zhong, D., and Sancar, A., 2008. Purification and characterization of a type III photolyase from *Caulobacter crescentus*. Biochemistry 47, 10255-10261.

Park, H.W., Kim, S.T., Sancar, A., and Deisenhofer, J., 1995. Crystal structure of DNA photolyase from *Escherichia coli*. Science 268, 1866-1894.

Passarelli, A.L., and Miller, L.K., 1994. Identification and transcriptional regulation of the baculovirus *lef-6* gene. J. Virol. 68, 4458-4467.

Pearson, M.N., Russell, R.L.Q., Rohrmann, G.F., and Beaudreau, G.S., 1988. P39, A major baculovirus structural protein: immunocytochemical characterization and genetic location. Virology 167, 407-413.

Peng, J., Elias, J.E., Thoreen, C.C., Licklider, L.J., and Gygi, S.P., 2003. Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: The yeast proteome. J. Proteome Res. 2, 43-50.

Peng, K., van Oers, M.M., Hu, Z., van Lent, J.W.M., and Vlak, J.M., 2010a. Baculovirus *per os* infectivity factors form a complex on the surface of occlusion derived virus (submitted).

Peng, K., Wu, M., Deng, F., Song, J., Dong, C., Wang, H., and Hu, Z., 2010b. Identification of protein-protein interactions of the occlusion-derived virus-associated proteins of *Helicoverpa armigera* nucleopolyhedrovirus. J. Gen. Virol. 91, 659-670.

Perera, O., Green, T.B., Stevens Jr, S.M., White, S., and Becnel, J.J., 2007. Proteins associated with C*ulex nigripalpus* nucleopolyhedrovirus occluded virions. J. Virol. 81, 4585-4590.

Pham, D.Q.D., Hice, R.H., Sivasubramanian, N., and Federici, B.A., 1993. The 1629-bp open reading frame of the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus encodes a virion structural protein. Gene 137, 275-280.

Pijlman, G.P., Pruijssers, A.J.P., and Vlak, J.M., 2003a. Identification of *pif-2*, a third conserved baculovirus gene required for *per os* infection of insects. J. Gen. Virol. 84, 2041-2049.

Pijlman, G.P., van Schijndel, J.E., and Vlak, J.M., 2003b. Spontaneous excision of BAC vector sequences from bacmid-derived baculovirus expression vectors upon passage in insect cells. J. Gen. Virol. 84, 2669-2678.

Pijlman, G.P., Vrij, J.d., End, F.J.v.-d., Vlak, J.M., and Martens, D.E., 2004. Evaluation of baculovirus expression vectors with enhanced stability in continuous cascaded insect-cell bioreactors. Biotechnol. Bioeng. 87, 743-753.

Räty, J.K., Airenne, K.J., Marttila, A.T., Marjomäki, V., Hytönen, V.P., Lehtolainen, P., Laitinen, O.H., Mähönen, A.J., Kulomaa, M.S., and Ylä-Herttuala, S., 2004. Enhanced gene delivery by avidin-displaying baculovirus. Mol. Ther. 9, 282-291.

Rauth, A.M., 1965. The physical state of viral nucleic acid and the sensitivity of viruses to ultraviolet light. Biophys. J. 5, 257-273.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208-212.

Rohrmann, G.F., 1986. Polyhedrin structure. J. Gen. Virol. 67, 1499-1513.

Rohrmann, G.F., 2008a. Baculovirus molecular biology. Bethesda (MD).

Rohrmann, G.F., 2008b. The baculovirus replication cycle: Effects on cells and insects. In Baculovirus Molecular Biology, pp. 29-44. Bethesda (MD).

Rohrmann, G.F., 2008c. DNA replication and genome processing, In Baculovirus Molecular Biology. pp. 55-70. Bethesda (MD).

Roth, D.M., Moseley, G.W., Glover, D., Pouton, C.W., and Jans, D.A., 2007. A microtubule-facilitated nuclear import pathway for cancer regulatory proteins. Traffic 8, 673-686.

Russell, R.L.Q., and Rohrmann, G.F., 1993. A 25-kDa protein is associated with the envelopes of occluded baculovirus virions. Virology 195, 532-540.

Russell, R.L.Q., Funk, C.J., and Rohrmann, G.F., 1997. Association of a baculovirus-encoded protein with the capsid basal region. Virology 227, 142-152.

Russell, R.L.Q., and Rohrmann, G.F., 1997. Characterization of P91, a protein associated with virions of an *Orgyia pseudotsugata* baculovirus. Virology 233, 210-223.

Rycyna, R.E., and Alderfer, J.L., 1985. UV irradiation of nucleic acids: formation, purification and solution conformational analysis of the '6-4 lesion' of dTpdT. Nucleic Acids Res. 13, 5949-5963.

Sancar, A., 1994. Structure and function of DNA photolyase. Biochemistry 33, 2-9.

Sancar, A., 1996. DNA excision repair. Annu. Rev. Biochem. 65, 43-81.

Sancar, A., 2003. Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. Chem. Rev. 103, 2203-2237.

References

Sancar, A., and Rupert, C.S., 1978. Cloning of the *phr* gene and amplification of photolyase in *Escherichia coli.* Gene 4, 295-308.

Sancar, A., and Sancar, G.B., 1984. *Escherichia coli* DNA photolyase is a flavoprotein. J. Mol. Biol. 172, 223-227.

Sancar, G.B., 1990. DNA photolyases: Physical properties, action mechanism, and roles in dark repair. DNA Repair 236, 147-160.

Sauer, G., Korner, R., Hanisch, A., Ries, A., Nigg, E.A., and Sillje, H.H.W., 2005. Proteome Analysis of the Human Mitotic Spindle. Mol. Cell. Proteomics 4, 35-43.

Schul, W., Jans, J., Rijksen, Y.M.A., Klemann, K.H.M., Eker, A.P.M., de Wit, J., Nikaido, O., Nakajima, S., Yasui, A., Hoeijmakers, J.H.J., and van der Horst, G.T.J., 2002. Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice. EMBO J. 21, 4719-4729.

Schulz, G.E., 1992. Binding of nucleotides by proteins. Curr. Opin. Struct. Biol. 2, 61-67.

Setlow, R.B., and Carrier, W.L., 1966. Pyrimidine dimers in ultraviolet-irradiated DNA's. J. Mol. Biol. 17, 237-254.

Slack, J., and Arif, B.M., 2006. The baculoviruses occlusion-derived virus: virion structure and function. Adv. Virus Res. 69, 99-165.

Slamovits, C.H. and Keeling, P.J., 2004. Class II photolyase in a microsporidian intracellular parasite. J. Mol. Biol. 341, 713-721.

Slaper, H., Velders, G.J.M., Daniel, J.S., de Gruijl, F.R., and van der Leun, J.C., 1996. Estimates of ozone depletion and skin cancer incidence to examine the Vienna Convention achievements. Nature 384, 256-258.

Smith, G.E., and Summers, M.D., 1978. Analysis of baculovirus genomes with restriction endonucleases. Virology 89, 517-527.

Sodeik, B., Ebersold, M.W., and Helenius, A., 1997. Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. J. Cell Biol. 136, 1007-1021.

Song, J., Wang, R., Deng, F., Wang, H., and Hu, Z., 2008. Functional studies of *per os* infectivity factors of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus. J. Gen. Virol. 89, 2331-2338.

Song, S.U., and Boyce, F.M., 2001. Combination treatment for osteosarcoma with baculoviral vector mediated gene therapy (p53) and chemotherapy (adriamycin). Exp. Mol. Med. 33, 46-53.

Srinivasan, V., Schnitzlein, W.M., and Tripathy, D.N., 2001. Fowlpox virus encodes a novel DNA repair enzyme, CPD-photolyase, that restores infectivity of UV light-damaged virus. J. Virol. 75, 1681-1688.

Srinivasan, V., and Tripathy, D.N., 2005. The DNA repair enzyme, CPD-photolyase restores the infectivity of UV-damaged fowlpox virus isolated from infected scabs of chickens. Vet. Microbiol. 108, 215-223.

Stanbridge, L.J., Dussupt, V., and Maitland, N.J., 2003. Baculoviruses as vectors for gene therapy against human prostate cancer. J. Biomed. Biotechnol. 2003, 79-91.

Stanley, M.A., 2003. Progress in prophylactic and therapeutic vaccines for human papillomavirus infection. Expert Rev. Vaccines 2, 381-389.

Stewart, L.M.D., Hirst, M., Ferber, M.L., Merryweather, A.T., Cayley, P.J., and Possee, R.D., 1991. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. Nature 352, 85-88.

Sun, X.L., Zhang, G.Y., Zhang, Z.X., Hu, Z.H., Vlak, J.M., and Arif, B.M., 1998. *In vivo* cloning of *Helicoverpa armigera* single nucleocapsid nuclear polyhedrosis virus genotypes. Virol. Sin. 13, 83-88.

Sun, X., Chen, X., Zhang, Z., Wang, H., Bianchi, F.J.J.A., Peng, H., Vlak, J.M., and Hu, Z., 2003. Bollworm responses to release of genetically modified *Helicoverpa armigera* nucleopolyhedroviruses in cotton. J. Invertebr. Pathol. 81, 63-69.

Sun, X., Sun, X., van der Werf, W., Vlak, J.M., and Hu, Z., 2004a. Field inactivation of wild-type and genetically modified *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus in cotton. Biocontrol Sci. Technol. 14, 185-192.

Sun, X., Wang, H., Sun, X., Chen, X., Peng, C., Pan, D., Jehle, J.A., van der Werf, Vlak, J.M., and Hu, Z., 2004b. Biological activity and field efficacy of a genetically modified *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus expressing an insect-selective toxin from a chimeric promoter. Biol. Control 29, 124-137.

Sun, X.L., and Peng, H.Y., 2007. Recent advances in biological control of pest insect by using viruses in China. Virol. Sin. 22, 158-162.

Suzuki, N., Nonaka, H., Tsuge, Y., Inui, M., and Yukawa, H., 2005. New multiple-deletion method for the *Corynebacterium glutamicum* genome, using a mutant lox sequence. Appl. Environ. Microbiol. 71, 8472-8480.

Szewczyk, B., Hoyos-Carvajal, L., Paluszek, M., Skrzecz, I., and Lobo de Souza, M., 2006. Baculoviruses -- re-emerging biopesticides. Biotechnol. Adv. 24, 143-160.

Tamada, T., Kitadokoro, K., Higuchi, Y., Inaka, K., Yasui, A., De Ruiter, P.E., Eker, A.P.M., and Miki, K., 1997. Crystal structure of DNA photolyase from *Anacystis nidulans*. Nat. Struct. Biol. 4, 887-891.

Theilmann, D.A., and Stewart, S., 1993. Analysis of the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus trans-activators IE-1 and IE-2 using monoclonal antibodies. J. Gen. Virol. 74, 1819-1826.

Thiem, S.M., and Cheng, X.W., 2009. Baculovirus host-range. Virol. Sin. 24, 436-457.

Tomalski, M.D., Eldridge, R., and Miller, L.K., 1991. A baculovirus homolog of a Cu/Zn superoxide dismutase gene. Virology 184, 149-161.

Trimble, C.L., and Frazer, I.H., 2009. Development of therapeutic HPV vaccines. Lancet Oncol. 10, 975-980.

Ueda, T., Kato, A., Kuramitsu, S., Terasawa, H., and Shimada, I., 2005. Identification and characterization of a second chromophore of DNA photolyase from *Thermus thermophilus* HB27. J. Biol. Chem. 280, 36237-36243.

Urabe, M., Ding, C., and Kotin, R.M., 2002. Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum. Gene Ther. 13, 1935-1943.

Vail, P.V., Sutter, G., Jay, D.L., and Gough, D., 1971. Reciprocal infectivity of nuclear polyhedrosis viruses of the cabbage looper and alfalfa looper. J. Invertebr. Pathol. 17, 383-388.

Vail, P.V., Hostetter, D.L., and Hoffmann, D.F., 1999. Development of the Multi-nucleocapsid Nucleopolyhedroviruses (MNPVs) Infectious to Loopers (Lepidoptera: Noctuidae: Plusiinae) as Microbial Control Agents. Int. Pest Manage. Rev. 4, 231-257.

van der Horst, G.T.J., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S.I., Takao, M., de Wit, J., Verkerk, A., Eker, A.P.M., van Leenen, D., Buijs, R., Bootsma, D., Hoeijmakers, J.H.J., and Yasui, A., 1999. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398, 627-630.

References

van Houten, B., 1990. Nucleotide excision repair in *Escherichia coli*. Microbiol. Mol. Biol. Rev. 54, 18-51.

van Lent, J.W.M., Groenen, J.T.M., Klinge-Roode, E.C., Rohrmann, G.F., Zuidema, D., and Vlak, J.M., 1990. Localization of the 34 kDa polyhedron envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. Arch. Virol. 111, 103-114.

van Oers, M.M., 2006. Vaccines for viral and parasitic diseases produced with baculovirus vectors. Adv. Virus Res. 68, 193-253.

van Oers, M.M., Herniou, E.A., Usmany, M., Messelink, G.J., and Vlak, J.M., 2004. Identification and characterization of a DNA photolyase-containing baculovirus from *Chrysodeixis chalcites*. Virology 330, 460-470.

van Oers, M.M., Abma-Henkens, M.H., Herniou, E.A., de Groot, J.C., Peters, S., and Vlak, J.M., 2005. Genome sequence of *Chrysodeixis chalcites* nucleopolyhedrovirus, a baculovirus with two DNA photolyase genes. J. Gen. Virol. 86, 2069 - 2080.

van Oers, M.M., and Vlak, J.M., 2007. Baculovirus genomics. Current Drug Targets 8, 1051-1068.

van Oers, M.M., Lampen, M.H., Bajek, M.I., Vlak, J.M., and Eker, A.P.M., 2008. Active DNA photolyase encoded by a baculovirus from the insect *Chrysodeixis chalcites*. DNA Repair 7, 1309-1318.

Vanarsdall, A.L., Okano, K., and Rohrmann, G.F., 2006. Characterization of the role of very late expression factor 1 in baculovirus capsid structure and DNA processing. J. Virol. 80, 1724-1733.

Vanarsdall, A.L., Pearson, M.N., and Rohrmann, G.F., 2007. Characterization of baculovirus constructs lacking either the Ac101, Ac142, or the Ac144 open reading frame. Virology 367, 187-195.

Vande Berg, B.J., and Sancar, G.B., 1998. Evidence for dinucleotide flipping by DNA photolyase. J. Biol. Chem. 273, 20276-20284.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J., and McCawley, P., 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). In Vitro 13, 213-217.

Vink, A.A., and Roza, L., 2001. Biological consequences of cyclobutane pyrimidine dimers. J. Photochem. Photobiol. B: Biol. 65, 101-104.

Vlak, J.M., 2007. Professor Shang yin Gao (1909-1989): His legacy in insect cell culture and insect virology. J. Invertebr. Pathol. 95, 152-160.

Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klinge-Roode, E.C., Geervliet, J.B.F., Roosien, J., and van Lent, J.W.M., 1988. Functional studies on the *p10* gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a *p10- beta - galactosidase* fusion gene. J. Gen. Virol. 69, 765-776.

Volkman, L.E., 2007. Baculovirus infectivity and the actin cytoskeleton. Curr. Drug Targets 8, 1075-1083.

Wakatsuki, T., Schwab, B., Thompson, N.C., and Elson, E.L., 2001. Effects of cytochalasin D and latrunculin B on mechanical properties of cells. J. Cell Sci. 114, 1025-1036.

Wang, G., Zhang, C., Gong, C., Jin, W., and Wu, X., 1997. Cloning and sequencing of *Helicoverpa armigera* nuclear polyhedrosis virus polyhedrin gene. Chin. J. Virol.13, 83-87.

Wang, H., Deng, F., Pijlman, G.P., Chen, X., Sun, X., Vlak, J.M., and Hu, Z., 2003. Cloning of biologically active genomes from a *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus isolate by using a bacterial artificial chromosome. Virus Res. 97, 57-63.

Wang, Y., Choi, J., Roh, J., Woo, S., Jin, B., and Je, Y., 2008. Molecular and phylogenetic characterization of *Spodoptera litura* granulovirus. J. Microbiol. 46, 704-708.

Wang, Y., Wu, W., Li, Z., Yuan, M., Feng, G., Yu, Q., Yang, K. and Pang, Y., 2007. Ac18 is not essential for the propagation of *Autographa californica* multiple nucleopolyhedrovirus. Virology 367, 71-81.

Ward, V.K., Sneddon, K.M.B., Hyink, O., and Kalmakoff, J., 2003. Baculovirus genomics: a resource for biological control. In: R.K. Upadhyay (Ed), Advances in microbial control of insect pests, pp. 127-143. Kluwer Academic/Plenum Publishers., New York, USA.

Weber, S., 2005. Light-driven enzymatic catalysis of DNA repair: a review of recent biophysical studies on photolyase. BBA-Bioenergetics 1707, 1-23.

Whittaker, G.R., Kann, M., and Helenius, A., 2000. Viral entry into the nucleus. Annu. Rev. Cell Dev. Biol. 16, 627-651.

Willer, D.O., McFadden, G., and Evans, D.H., 1999. The complete genome sequence of Shope (rabbit) fibroma virus. Virology 264, 319-343.

Williams, G.V., and Faulkner, P., 1997. Cytological changes and viral morphogenesis during baculovirus infection. In: L.K. Miller (Ed), The baculoviruses. Plenum Press, New York, USA.

Willis, L.G., Siepp, R., Stewart, T.M., Erlandson, M.A., and Theilmann, D.A., 2005. Sequence analysis of the complete genome of *Trichoplusia ni* single nucleopolyhedrovirus and the identification of a baculoviral photolyase gene. Virology 338, 209-226.

Wilson, M.E., and Price, K.H., 1988. Association of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) with the nuclear matrix. Virology 167, 233-241.

Wood, H.A., and Granados, R.R., 1991. Genetically engineered baculoviruses as agents for pest control. Annu. Rev. Microbiol. 45, 69-87.

Wu, W., Lin, T., Pan, L., Yu, M., Li, Z., Pang, Y., and Yang, K., 2006. *Autographa californica* multiple nucleopolyhedrovirus nucleocapsid assembly is interrupted upon deletion of the *38K* gene. J. Virol. 80, 11475-11485.

Xu, F., Vlak, J.M., and van Oers, M.M., 2008. Conservation of DNA photolyase genes in group II nucleopolyhedroviruses infecting plusiine insects. Virus Res. 136, 58-64.

Xu, F., Vlak, J.M., Eker, A.P.M., and van Oers, M.M., 2010. DNA photolyases of *Chrysodeixis chalcites* nucleopolyhedrovirus are targeted to the nucleus and interact with chromosomes and mitotic spindle structures. J. Gen. Virol. 91, 907-914.

Yasui, A., Eker, A.P.M., Yasuhira, S., Yajima, H., Kobayashi, T., Takao, M., and Oikawa, A., 1994. A new class of DNA photolyases present in various organisms including aplacental mammals. EMBO J. 13, 6143-6151.

Yasui, A., and McCready, S.J., 1998. Alternative repair pathways for UV-induced DNA damage. BioEssays 20, 291-297.

Zhang, G., Zhang, Y., Ge, L., and Shan, Z., 1981. The production and application of the nuclear polyhedrosis virus of *Heliothis armigera* (Hübner) in biological control. Acta Phytophylactica Sinica 8, 235-240.

Zhu, S.-Y., Yi, J.-P., Shen, W.-D., Wang, L.-Q., He, H.-G., Wang, Y., Li, B., and Wang, W.-B., 2009. Genomic sequence, organization and characteristics of a new nucleopolyhedrovirus isolated from *Clanis bilineata* larvae. BMC Genomics 10, 91.

References

Zwart, M.P., Erro, E., van Oers, M.M., de Visser, J.A.G.M., and Vlak, J.M., 2008a. Low multiplicity of infection *in vivo* results in purifying selection against baculovirus deletion mutants. J. Gen. Virol. 89, 1220-1224.

Zwart, M.P., van Oers, M.M., Cory, J.S., van Lent, J.W.M., van der Werf, W., and Vlak, J.M., 2008b. Development of a quantitative real-time PCR for determination of genotype frequencies for studies in baculovirus population biology. J. Virol. Methods 148, 146-154.

List of abbreviations

AAV	Adeno-associated virus
8-HDF	8-hydroxy-7,8-didemethyl-5-deazaflavin
ABC	Ammonium bicarbonate
ACN	Acetonitrile
AMEV	*Amsacta moorei* entomopoxvirus
BCA	Bicinchoninic acid
BER	Base excision repair
BEVS	Baculovirus expression vector system
bp	Base pair
BSA	Bovine serum albumin
BV	Budded virus
Cc-PHR1	ChchNPV encoded photolyase 1
Cc-PHR2	ChchNPV encoded photolyase 2
cDNA	copy Deoxyribonucleic acid
CPD	Cyclobutane pyrimidine dimer
CPE	Cytopathological effect
cry	Cryptochrome
CSF	Classical swine fever
CV	Column volume
EGFP	Enhanced green fluorescent protein
EGT	Ecdysteroid UDP-glucosyltransferase
EM	Electron microscopy
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FMN	Flavin mononucleotide
FTMS	Fourier transform mass spectrometry
FWPV	Fowlpox virus
GT	Guatemala
GV	Granulovirus
ie2	Immediate early 2 gene
IgG	Immunoglobulin G
LC-MS/MS	Liquid chromatography-linked tandem mass spectrometry
MALDI-TOF MS	Matrix-assisted laser desorption ionization time of flight mass spectrometry
MNPV	Multiple nucleopolyhedrovirus
MOI	Multiplicity of infection
MSEV	*Melanoplus sanguinipes* entomopoxvirus
MTHF	5,10-methenyltetrahydrofolate
MW	Molecular weight markers
MYXV	Myxomavirus
NCBI	National Center for Biotechnology Information
NER	Nucleotide excision repair
NJ	Neighbor-joining
NL	Netherlands/Dutch
NPV	Nucleopolyhedrovirus
OB	Occlusion body
ODV	Occlusion-derived virus

Abbreviations

ORF	Open reading frame
p.i.	Post infection
p.t.	Post transfection
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Polyhedrin envelope protein
phr	DNA photolyase gene
PIF	*Per os* infectivity factor
polh	Polyhedrin gene
qPCR	Quantitative real-time PCR
rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFV	Shope fibroma virus
SNPV	Single nucleopolyhedrovirus
SOD	Superoxide dismutase
SP	Spanish
TFA	Triflouroacetic acid
UV	Ultraviolet
VLP	Virus-like particle
(6-4)PP	Pyrimidine–pyrimidone (6-4) photoproduct

Summary

The baculoviruses (Family *Baculoviridae*) form a group of large, enveloped DNA viruses which are highly pathogenic and often cause fatal disease in insect larvae, predominantly in insects from the order Lepidoptera. Baculoviruses are attractive as biological agents to control pest insects, as these viruses are virulent, often host specific and safe for the environment. Solar radiation is regarded as a major factor that negatively affects the field performance of baculoviruses. Inactivation by UV light plays an important role in baculovirus ecology and has limited the expansion of baculoviruses as biological insecticides.

When DNA is exposed to UV light, adjacent pyrimidines within the same DNA strand may form cyclobutane pyrimidine dimers (CPD) and pyrimidine–pyrimidone (6-4) photoproducts at a ratio of about 3:1 *in vivo*. Both photoproducts are cytotoxic as they block transcription, induce mutations and trigger apoptosis. CPDs and (6-4) photoproducts can be repaired by specific photolyase, DNA repair enzymes, which use visible light as energy source. CPD photolyases are divided into two classes based on their amino acid sequence divergence. All CPD photolyases carry a catalytic FAD co-factor and they also contain a light-harvesting co-factor. Photolyases are presents in all groups of organisms except in placental mammals. Class II CPD photolyase (*phr*) genes have been identified in baculoviruses isolated from the tomato looper (*Chrysodeixis chalcites* nucleopolyhedrovirus, ChchNPV) and the cabbage looper, *Trichoplusia ni* (TnSNPV), both belonging to the Plusiinae subfamily of the Noctuidae. Functional assays, in *E. coli* and *in vitro,* showed that *Cc-phr2* encodes an active CPD photolyase. The study described in this thesis focuses on the prevalence of photolyase genes within the Plusiinae subfamily, the intracellular localization of the two Cc-PHRs proteins in insect cells and their presence in the proteome of the ChchNPV occlusion derived virus (ODV). To show whether photolyases are active in a baculovirus context, the UV sensitivity of a recombinant baculovirus carrying CcPHR2 was also studied.

In Chapter 2, a PCR-based strategy was developed to find possible photolyase gene homologs in Plusiinae-infecting NPVs. Six additional Plusiinae-infecting NPVs were analyzed and all, except the *Thysanoplusia oricalcea* NPV strain A28-1, contained one or more *phr*-like sequences. Phylogenetic analysis revealed that all photolyase genes of the tested Plusiinae-infecting baculoviruses group in a single clade, which separates into three subgroups. The phylogeny of the polyhedrin sequences confirmed that the viruses that contain *phr* genes are group II NPVs, but that *Thysanoplusia oricalcea* NPV A28-1, which lacks such a gene, belongs to NPV group I. It is hypothesized that all plusiine group II NPVs may contain one or more photolyase genes derived from a common ancestor.

A CPD photolyase with biological activity should have the ability to bind DNA. Since baculoviruses replicate and assemble in the nucleus of insect cells, PHR proteins need to be targeted to the nucleus to be able to interact with baculovirus DNA. Both PHR1 and PHR2 were fused to EGFP and both fusion proteins localized in the nucleus in transient expression assays. For non-fused PHR2 a nuclear localization early after transfection could also be shown by immunofluorescence studies. Both PHR-EGFP fusion proteins strongly colocalized with chromosomes and with spindle, aster and midbody structures during insect cell mitosis. When *phr2-egfp*-transfected cells were superinfected with *Autographa californica* (Ac) MNPV, the PHR2-EGFP protein colocalized with virogenic stroma,

where viral replication takes place and where large amounts of viral DNA are present. The interaction of both viral PHR proteins with chromosomes and with the virogenic stroma as observed for PHR2 suggest that these proteins can bind to condensed DNA, a situation that exists in baculovirus nucleocapsids, both in incoming parental virions and in assembling progeny nucleocapsids. The observations presented in Chapter 3 support the supposition that the PHR2 protein plays a role in baculovirus DNA repair.

No PHR2 or PHR2-EGFP was found in viral occlusion bodies (OBs) isolated from the cells that were transfected and subsequently infected with AcMNPV by Western blot analysis. Moreover, Cc-PHR2 was not detectable in ODV nor in budded virus (BV) of ChchNPV by immuno-detection. In Chapter 4 the LC-MS/MS approach provided a sensitive method to determine whether Cc-PHR proteins are present in the proteome of ChchNPV ODVs. Fifty-three viral proteins were identified in ChchNPV ODVs, but both PHR proteins were not detected. One ODV-protein is encoded by a gene so far unique to ChchNPV (*Chch105*). A comparison of the ODV proteins identified in ChchNPV and in the ODV proteomes of two other alpha- and one deltabaculovirus, showed ten conserved ODV proteins (ODV-E18, ODV-E56, ODV-EC27, ODV-EC43, P6.9, P33, P49, P74, GP41, and VP39). In addition, PIF-1, PIF-2, and PIF-3, all found in the ChchNPV ODV proteome, should be considered as conserved ODV proteins as they have been recognized as ODV envelop proteins in other studies. Twenty-two proteins which have not been identified as ODV proteins in previous studies were found in ChchNPV ODVs despite the presence of homologous genes in the other viral genomes.

In order to test the activity of ChchNPV PHR2 in a baculovirus background, the UV sensitivity of *Helicoverpa armigera* NPV (HearNPV) endowed using bacmid technology with the *Cc-phr2* gene was tested and compared with a 'wild type'HearNPV lacking this gene in a bioassay (Chapter 5). A three-fold increase in mortality was found for HearNPV carrying the ChchNPV *phr* gene as compared to wild type HearNPV, when tested in cotton bollworm larvae. This increase was found despite the fact that only a small proportion of the viral genome copies contained the *Cc-phr2* ORF. PCR analyses indicated that a high amount of wild-type HearNPV genomes were present in the bacmid-derived virus, which probably resulted from genome instability of the HearNPV recombinant bacmids. The obtained data support the hypothesis that the Cc-*phr2* gene gives a baculovirus enhanced tolerance to UV light. It may give plusiine baculoviruses an ecological benefit, as *phr* genes make the virus less sensitive to UV.

A cell line, named WU-CcE-1, was established from embryos of the tomato looper *Chrysodeixis chalcites* (Chapter 6). The cell line was distinguished from three other lepidopteran cell lines propagated in the laboratory by DNA amplification fingerprinting. The cultured CcE-1 cells were permissive for infection by ChchNPV as well as TnSNPV. In contrast, AcMNPV and *HearNPV* both induced apoptosis in these cells. This novel cell line will be a useful tool for the generation of a ChchNPV bacmid and the production of *phr* deletion viruses to further study the role of Cc-PHRs in ChchNPV biology.

In conclusion, the research described in this thesis revealed that *phr* genes are a typical feature of group II NPVs that infect noctuids belonging to the subfamily Plusiinae. The ChchNPV encoded CPD photolyases targeted to the nucleus and associated with chromosomes in transient expression assays and with virogenic stroma (PHR2) upon virus infection. Both Cc-PHR proteins were not present in detectable amounts in ChchNPV ODV particles, where they were expected to be located to photoreactivate damaged DNA prior to viral gene expression. This observation complicates our understanding of the potential role of *phr* genes in the infection cycle of plusiine baculoviruses. Despite the fact that PHR2 was not detected in ODVs by LC/MS-MS, incorporation of the *phr2* gene

in the baculovirus HearNPV led to blue light-dependent reduction in UV sensitivity of this virus. A new cell line was established that showed permissive replication of ChchNPV and greatly facilitates the further investigation of this virus and its *phr* genes. The role of photolyases in the replication of plusiine baculoviruses at the cellular, organismal and ecosystem level needs further clarification.

Samenvatting

Baculovirussen (Familie *Baculoviridae*) zijn grote DNA-bevattende virussen omgeven door een lipidenmembraan. Deze virussen zijn zeer pathogeen voor insecten en veroorzaken vaak fatale ziektes in larven van insecten, voornamelijk behorend tot de Orde Lepidoptera. Baculovirussen zijn geschikt om op biologische wijze plaaginsecten te bestrijden, omdat deze virussen virulent en specifiek zijn en veilig voor de omgeving. De werkzaamheid van baculovirussen in de praktijk wordt echter negatief beïnvloed door zonlicht, met name door de UV component. Inactivatie van het virus door UV licht heeft er toe bijgedragen dat baculovirussen niet op grote schaal kunnen worden ingezet als biologisch insecticide, tenzij UV-beschermingsmiddelen worden toegevoegd.

Wanneer DNA wordt blootgesteld aan UV licht, kunnen naast elkaar liggende pyrimidinebasen in dezelfde DNA streng cyclobutaan-pyrimidine dimeren (CPD) en pyrimidine–pyrimidon (6-4 PP) fotoprodukten vormen, die *in vivo* voorkomen in een verhouding van ongeveer 3:1. Beide typen fotoprodukt zijn cytotoxisch, doordat ze transcriptie blokkeren, mutaties induceren en cellen aanzetten tot apoptose. CPDs en 6-4 PPs kunnen worden gerepareerd door specifieke enzymen genaamd fotolyases, DNA reparatie-enzymen die zichtbaar licht gebruiken als energiebron. CPD fotolyases worden onderverdeeld in twee klassen op basis van hun aminozuurvolgorde. Alle CPD fotolyases binden een katalytische FAD cofactor en een lichtafhankelijke cofactor. Photolyases zijn aanwezig bij alle groepen organismen behalve bij placentale zoogdieren.

CPD fotolyase (*phr*) genen van klasse II zijn niet zo lang geleden ook geïdentificeerd in baculovirussen, en wel in baculovirussen die geïsoleerd zijn uit larven van de Turkse mot (*Chrysodeixis chalcites* nucleopolyhedrovirus, ChchNPV) en de koolmot of ni-uil, *Trichoplusia ni* (TnSNPV). Beide insecten behoren tot de onderfamilie Plusiinae van de Noctuidae. Studies in de bacterie *Escherichia coli* en *in vitro* hebben reeds laten zien dat één van de twee ChchNPV *phr* genen (*Cc-phr2*) codeert voor een actief CPD fotolyase. De centrale thema's van de in dit proefschrift beschreven studie zijn: het vóórkomen van fotolyase genen in andere baculovirussen van insecten in de Plusiinae onderfamilie, de intracellulaire locatie van de twee Cc-PHR eiwitten in insectencellen en hun mogelijke aanwezigheid in het proteoom van ChchNPV. Ook werd de UV gevoeligheid van baculovirussen mét en zonder fotolyasegen bestudeerd om te toetsen of fotolyases functioneel zijn in een baculovirusomgeving.

In Hoofdstuk 2 werd een PCR methode ontwikkeld om eventuele CPD fotolyasegenen te detecteren in baculovirussen (NPVs) die plusiine insecten infecteren. Daartoe werden zes niet eerder onderzochte Plusiinae-infecterende NPVs geanalyseerd en deze hadden alle, behalve *Thysanoplusia oricalcea* NPV stam A28-1, één of meer *phr*-achtige sequenties. Fylogenetische analyse liet zien dat alle fotolyasegenen van de geteste Plusiinae-infecterende baculovirussen samen één fylogenetisch cluster vormen, dat onderverdeeld is in drie subgroepen. De parallelle analyse van de polyhedrinesequenties liet zien, dat de virussen die *phr* genen bezitten behoren tot NPV groep II. *Thysanoplusia oricalcea* NPV isolaat A28-1 daarentegen,dat geen *phr* gen heeft behoort tot de NPVs groep I. Op basis hiervan is een hypothese opgesteld dat alle plusiine group II NPVs één of meer fotolyasegenen bevatten, die ook nog eens afkomstig zijn van een gemeenschappelijke voorouder.

Een CPD fotolyase met biologische activiteit zal in staat moeten zijn om DNA te binden. Omdat baculovirussen repliceren en assembleren in de kern van insectencellen, moeten PHR-eiwitten naar de

celkern getransporteerd worden om met baculovirus DNA in contact te komen en dit te kunnen repareren. Zowel PHR1 als PHR2 werd gefuseerd met EGFP en beide fusie-eiwitten gingen, in expressiestudies, naar de kern. Ook in niet-gefuseerde vorm werd PHR2 naar de kern getransporteerd, zoals bleek uit immuunfluorescentiestudies. Beide PHR-EGFP fusieproteïnen colocaliseerden met chromosomen, spoelfiguren en ster- en "midbody" structuren tijdens de celdeling (mitose) van insectencellen. Wanneer PHR2-EGFP-getransfecteerde cellen daarna werden geïnfecteerd met *Autographa californica* (Ac) MNPV, colocaliseerde het fusie-eiwit met het virogene stroma. In dit stroma vindt virusreplicatie plaats en zijn grote hoeveelheden viraal DNA aanwezig. De interactie van de virale PHR eiwitten met chromosomen en met het virogene stroma, zoals waargenomen voor PHR2, suggereert, dat deze eiwitten kunnen binden aan gecondenseerd DNA. Deze situatie bestaat ook in nucleocapsiden van baculovirussen, zowel in virusdeeltjes die de cel in komen als in assemblerende nieuwe virions. De waarnemingen weergegeven in Hoofdstuk 3 ondersteunen de veronderstelling, dat het ChchNPV PHR2 eiwit een rol speelt in het herstellen van schade in DNA.

Met immunoblotanalyse konden PHR2 en PHR2-EGFP niet worden aangetroffen in virale insluitlichamen (polyeders), die geïsoleerd waren uit getransfecteerde en vervolgens met AcMNPV geïnfecteerde cellen. Ook kon PHR2 niet gedetecteerd worden met immunodetectie in beide type virions, de extracelluaire virusdeeltjes en de ingesloten virusdeeltjes (ODV). In Hoofdstuk 4 werd van de gevoelige LC-MS/MS aanpak gebruik gemaakt om te bepalen of Cc-PHR eiwitten deel uitmaken van het proteoom van ChchNPV ODVs. In totaal werden drieënvijftig virale eiwitten geïdentificeerd in de ODVs van ChchNPV, waarvan één wordt gecodeerd door een gen dat tot nu toe alleen in ChchNPV (*Chch105*) gevonden is. Echter geen van de twee PHR eiwitten werd gevonden in het ODV proteoom. Een vergelijking van de ODV eiwitten van ChchNPV met het ODV proteoom van twee andere alfabaculovirussen en één deltabaculovirus liet zien dat tien ODV eiwitten in al deze proteomomen geconserveerd zijn (ODV-E18, ODV-E56, ODV-EC27, ODV-EC43, P6.9, P33, P49, P74, GP41, and VP39). Tevens moeten de drie eiwitten PIF-1, PIF-2, and PIF-3, die onmisbaar zijn voor orale infectie en die alle drie gevonden zijn in het ChchNPV ODV proteoom, beschouwd worden als geconserveerde ODV eiwitten, omdat als ODV membraaneiwitten zijn gevonden in andere studies. Tweeëntwintig eiwitten zijn hier voor het eerst gevonden in een ODV proteoom, ondanks de aanwezigheid van homologe genen in de andere virale genomen.

Om de activiteit van ChchNPV PHR2 te testen tegen een baculovirusachtergrond werd *Helicoverpa armigera* NPV (HearNPV) verrijkt met het *Cc-phr2* gen met behulp van bacmidentechnologie. De UV gevoeligheid van het recombinante HearNPV werd getest en in een bioassay vergeleken met het oorspronkelijke HearNPV, dat dit gen niet heeft (Hoofdstuk 5). De sterfte van katoenrupsen (*Helicoverpa armigera*) was driemaal hoger bij HearNPV mét *Cc-phr2* dan bij het 'wild type' HearNPV. Deze toename werd gevonden ondanks het feit dat slechts een klein deel van de virale genoomcopieën het Cc-*phr2* gen bleek te bevatten, zoals bleek uit PCR analyses. Hoogstwaarschijnlijk is dit een gevolg van genoominstabiliteit van recombinante HearNPV bacmiden. De verkregen data ondersteunen de hypothese dat het Cc- *phr2* gene de tolerantie van baculovirussen voor UV licht vergroot. Als gevolg daarvan zouden de plusiine baculovirussen ecologisch profijt kunnen hebben van de *phr* genen.

Voorts werd een cellijn met de naam WU-CcE-1 ontwikkeld uit embryo's van de Turkse mot *Chrysodeixis chalcites* (Hoofdstuk 6). De nieuwe cellijn kon met DNA amplificatie-vingerprinttechnieken onderscheiden worden van de drie andere cellijnen, die gelijktijdig in het laboratorium werden gegroeid. De CcE-1 cellen bleken vatbaar voor infectie met ChchNPV en TnSNPV. Daarentegen, veroorzaakte AcMNPV en *HearNPV apoptose* in deze cellen. Deze nieuwe

cellijn is een belangrijk instrument om ChchNPV varianten zonder *phr* genen te construeren ten einde de rol van de PHR-eiwitten in de ecologie van ChchNPV verder te kunnen onderzoeken. Ook kan deze cellijn gebruikt worden om een ChchNPV bacmide te maken voor fundamenteel onderzoek aan ChchNPV.

    Concluderend kan worden gesteld dat het onderzoek, dat in dit proefschrift beschreven is, heeft laten zien dat *phr* genen een karakteristieke eigenschap zijn van groep II NPVs van nachtvlinders in de onderfamilie Plusiinae. De twee CPD fotolyases, die gecodeerd worden door ChchNPV, zijn nucleaire eiwitten, die associëren met chromosomen en met het virogene stroma (PHR2) in baculovirus geïnfecteerde cellen. Beide Cc-PHR eiwitten zijn niet in detecteerbare hoeveelheid aanwezig in ChchNPV ODVs, waar ze wel verwacht werden ten einde beschadigd DNA te kunnen repareren vóór de start van virale genexpressie en replicatie. Deze waarneming maakt het lastiger om de rol van *phr* genen in de infectiecyclus van baculovirussen te begrijpen. Ondanks het feit dat PHR2 niet gedetecteerd is in ODVs met behulp van LC/MS-MS, leidde het inbouwen van een *phr2* gen in het baculovirus HearNPV tot een licht-afhankelijke afname van de UV gevoeligheid van dit virus. Tot slot werd een nieuwe cellijn ontwikkeld, waarin ChchNPV kan repliceren. Deze cellijn is een belangrijk hulpmiddel bij verder onderzoek aan dit virus en zijn *phr* genen. Het effect van fotolyases op de vermeerdering van plusiine baculovirussen (op cellulair, insect- en ecosysteemniveau) vergt nadere verheldering.

摘要

杆状病毒 （杆状病毒科）是一类有囊膜包被的大型 DNA 病毒，它们对于以鳞翅目为主的昆虫幼虫具有高度致病性并常引起幼虫死亡。 由于具有高效，专一并且对环境安全等特点，杆状病毒作为控制昆虫病害的生物杀虫剂具有很大潜力。 阳光辐射被认为是影响杆状病毒在田间杀虫效果的主要因素。紫外线（UV）辐射引起的病毒失活不仅对杆状病毒生态起着重要作用而且还限制了这些病毒杀虫剂的广泛使用。 DNA 在 UV 光的照射下，位于同一 DNA 链的相邻的两个嘧啶可以形成环丁烷嘧啶二聚体（CPD）和 嘧啶-嘧啶酮 （6-4）光化产物，两类光化产物生成的比率为 3:1。 这两种光化产物能够阻断转录，引起突变激发细胞凋亡，因而具有细胞毒性。 CPD 和（6-4）光化产物可以被特异的 DNA 修复酶-光解酶在可见光的条件下修复。 根据氨基酸序列的差异，CPD 光解酶可以分为两类。 所有的 CPD 光解酶都携带一个具有催化能力的 FAD 辅因子和一个可见光收集的辅辅助因子。 此酶广泛存在于除有胎盘的哺乳动物以外的所有生命体中。 类 II CPD 光解酶基因已在两种杆状病毒 *Chrysodeixis chalcites* nucleopolyhedrovirus 和 ChchNPV *Trichoplusia ni* (TnSNPV)中鉴定出来，它们分别感染夜蛾科（Noctuidae），金翅夜蛾亚科（Plusiinae）中的番茄尺蠖和粉纹夜蛾。 通过在大肠杆菌及体外的功能测试中发现 *Cc-phr2* 编码一个有活性的光解酶。 本论文着重研究了光解酶基因在金翅夜蛾亚科是否普遍存在， 两个 Cc-PHR 蛋白在昆虫细胞的亚细胞定位以及在包涵体病毒（ODV）中的定位。 为了证明光解酶在杆状病毒感染过程中是否具有活性，我们还对其 UV 敏感度进行了测定。

在第二章中，运用了基于 PCR 的策略在其他感染 Plusiinae 的核多角体病毒（NPV）中寻找光解酶的同源基因。 另外六个感染 Plusiinae 的 NPV，除 *Thysanoplusia oricalcea* NPV A28-1 以外，所有被检测的 NPV 都含有至少一个类似 *phr* 基因的序列。 进化分析揭示所有通过鉴定得到的这些光解酶基因形成一个进化枝，并且进一步分成三个子群。 以多角体蛋白序列建立的进化树证实了这些拥有 *phr* 基因的病毒属于组 II NPV，然而缺失此基因的 *Thysanoplusia oricalcea* NPV A28-1 却属于组 I NPV。 由此推测，所有组 II Plusiine NPV 包含至少一个同源的光解酶基因。

一个具有生物活性的 CPD 光解酶应该具有结合 DNA 的能力。 由于杆状病毒的复制及组装是在昆虫细胞的细胞核内完成的，因此 PHR 蛋白需要靶定细胞核来结合杆状病毒 DNA。 在瞬时表达实验中，分别与 EGFP 融合的 PHR1 和 PHR2 都定位于细胞核中。 免疫荧光实验也证实在转染后的早期，没有融合的 PHR2 定位在细胞核中。 在细胞有丝分裂期间，两个 PHR-EGFP 融合蛋白与染色体，纺锤体，星状体及中间体紧密结合。 在 PHR2-EGFP 转染细胞后用 *Autographa californica* (Ac) MNPV 进行超感染，发现融合蛋白定位于因病毒复制而出现大量新生 DNA 的病毒发生基质（virogenic stroma, VS）中。 病毒 PHR 蛋白与染色体以及 PHR2 与 VS 的相互作用暗示着这些蛋白可以结合到浓缩的 DNA 上，这与入侵的父代和装配中的子代病毒

摘要

核衣壳也结合于这种浓缩 DNA 的情况相似。 此章中的发现支持关于 PHR2 蛋白在杆状病毒 DNA 的修复过程中发挥功能的假说。

通过 Western blot 检测，在经过转染而后超级感染过的细胞分离出的病毒包涵体中没有发现 PHR2 或是 PHR2-EGFP 的存在。 此外免疫实验手段也没能在 ChchNPV 的 ODV 和出芽病毒（BV）中检测到 Cc-PHR2。 在第四章中，LC-MS/MS 提供了高灵敏度的手段来证明 Cc-PHR 蛋白是否存在于 ChchNPV ODV 的结构蛋白组分中。 总共五十三个 ChchNPV ODV 结构蛋白被鉴定出来，但没有发现这两个 Cc-PHR 蛋白。 其中编码 Chch105 蛋白的基因是 ChchNPV 独有的。 通过与两个 alpha- 和 一个 delta 杆状病毒 ODV 蛋白进行序列比对发现，十个保守的 ODV 蛋白(ODV-E18, ODV-E56, ODV-EC27, ODV-EC43, P6.9, P33, P49, P74, GP41 和 VP39)存在于 ChchNPV 结构蛋白中。 另外，鉴于 PIF-1, PIF-2 和 PIF-3 早已在以往的研究中被定义为 ODV 囊膜蛋白，在 ChchNPV ODV 蛋白组中发现的这三个蛋白也应该被视为保守的 ODV 蛋白。 二十二个在先前研究中没有被发现的 ODV 蛋白在 ChchNPV ODV 蛋白质组中首次被鉴定出来，尽管在其他病毒的基因组中能够找到同源基因。

运用 bacmid 重组技术（Bac-to-Bac, invitrogen）构建了表达 *Cc-phr2* 基因的 *Helicoverpa armigera*NPV (HearNPV) 重组病毒，而后通过与不含有 *Cc-phr2* 基因的野生型 HearNPV 对 UV 的敏感度比较来测试 ChchNPV PHR2 在杆状病毒系统中的活性。 相对于野生型 HearNPV，携带 ChchNPV *phr* 基因的重组病毒导致了棉铃虫死亡率增长了三倍，虽然只有一小部分病毒基因组包含 *Cc-phr2*。 PCR 分析说明在 bacmid 生成的病毒中存在相当一部分野生型 HearNPV 基因组，这也许是由于重组 bacmid 的基因组不稳定引起的。 生物测定的结果支持了 *Cc-phr2* 基因可以增强杆状病毒对于紫外光忍耐度这一假设得到了。 *phr* 基因对于病毒 UV 敏感度的降低也许会给 Plusiine 杆状病毒带来生态学上的优势。

以 *Chrysodeixis chalcites* 的胚胎为基础建立了一个名为 WU-CcE-1 的细胞系。 这个细胞系通过 DNA 指纹图谱的识别，可以与实验室其他三个培养的鳞翅目的细胞系区分开来。 经过培养的 CcE-1 细胞可以支持 ChchNPV 和 TnSNPV 病毒的感染。 相反的，AcMNPV 和 HearNPV 却引起 CcE-1 细胞的凋亡。 通过这个新建立的细胞系构建 ChchNPV bacmid 以及进一步构建 *phr* 缺失的重组病毒对于今后研究 Cc-PHR 在 ChchNPV 生物学上的作用是非常有帮助的。

综上所述，本研究揭示了 *phr* 基因是组 II Plusinne NPV 的典型特征。 由 ChchNPV 编码的 CPD 光解酶在瞬时表达实验中可以靶定到细胞核并且与染色体结合，在病毒感染的条件下 PHR2 能与 virogenic stroma 发生结合。 然而 Cc-PHR 原本预期定位在 ODV 中以便在病毒基因表达之前来光修复受损的 DNA，但在 ChchNPV ODV 包涵体中都没能检测到可测数量两种 Cc-PHR。 这对于在探究 *Cc-phr2* 基因在杆状病毒生活史中的潜在作用上变得错综复杂。 尽管 LC-MS/MS 没能在 ODV 中检测到 PHR2，引入了 *phr2* 基因的 HearNPV 减少了此重组病毒对于紫外光的敏感度。 新建立的细胞系支持 ChchNPV 病毒的复制，极大地利于这个病毒和 *phr* 基因的进一步研究。 光解酶在 Plusiine 杆状病毒复制过程中发挥的作用需要在细胞，组织和生态系统水平上进行更深入地阐明。

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have learned a lot from you about virus production, isolation and bioassay design. During my stay in Wuhan, I obtained help and support from many people, Dr. Deng Fei, Prof. Wang Hualin, Prof. Chen Xinwen, Dr. Yin Feifei, Shen Shu and He Yu. I would like to give my great thanks to Dr. Wang Manli. Manli, you are my best friend in the scientific field and I am so lucky that I met you during my PhD. I learnt special experimental tricks from you and we exchanged many ideas on research and life outside the laboratory. You always encouraged me when I was down. Thanks for your mom's delicious food that made me feel at home.

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Curriculum Vitae

Fang Xu (徐方) was born on the 24th of October, 1978, in Tianjin, China. In the years 1997-2001 she studied General Biology at Tianjin Normal University. After she obtained her Bachelor degree from this university, she worked as teacher in Biology in a high school in Tianjin. In September 2003, she started her MSc study Biotechnology at Wageningen University, The Netherlands. From September 2004 to October 2005, she performed a major thesis "Oral immunization of carp with plant derived LTB-linked *Spring viraemia of carp virus* (SVCV) G protein" at the Cell Biology and Immunology Group, Wageningen University and a minor thesis at the Netherlands Vaccine Institute (NVI), entitled "Construction and characterization of recombinant respiratory syncytial viruses harbouring the EGFP gene and its rescue in monkey kidney Vero cells." In November 2005, she received her Master degree in Biotechnology, specializing in Cellular and Molecular Science from Wageningen University. In December 2005, she started her PhD studies at the Laboratory of Virology, Wageningen University in the Joint PhD training Programme of the Chinese Academy of Sciences and the Royal Netherlands Academy of Science. She performed part of her PhD studies at the Wuhan Institute of Virology, Hubei Province, China, where she stayed for three months in 2009. She performed research on the DNA photolyases from *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) which resulted in this thesis.

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)
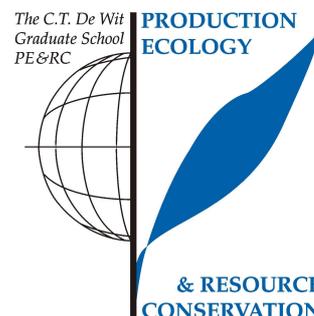
Review of literature (6 ECTS)
- Biology of baculovirus and UV-induced DNA repair; presented at CAS-KNAW Joint PhD Training Programme Event (2006)

Post-graduate courses (10.5 ECTS)
- Structure and dynamics of biomolecules *in vivo* en *in vitro* (2006)
- Biology underpinning animal sciences: Broaden your horizon (2007)
- Confocal light microscopy: fundamental and biological applications (2007)
- Basic & advanced statistics (2008)
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Laboratory training and working visits (4.5 ECTS)
- Bioassay of recombinant HearNPV against UV irradiation; Wuhan Institute of Virology, China (2009)

Competence strengthening / skills courses (2.7 ECTS)
- Information literacy for PhD + EndNote introduction (2007)
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PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
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- CAS-KNAW Joint PhD Training Programme Event (2006, 2008)
- Dutch annual virology symposia (2006-2010)
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- Programme Strategic Scientific Alliances (PSA)- local progress meeting (2009)

International symposia, workshops and conferences (8.6 ECTS)
- The 39th annual meeting of society for invertebrate pathology with oral presentation (2006)
- The 41th annual meeting of society for invertebrate pathology with oral presentation (2008)
- The first annual meeting of national key research and development project 973 of China (2009CB118903) with oral presentation (2009)
- Programme Strategic Scientific (PSA) – international workshop (2010)

Lecturing / supervision of practical's / tutorials: how to explain science to students and leadership (7.2 ECTS)
- Cell Biology and Health (2006-2009)

Supervision of MSc students: organising capacity, creativeness, problem solving capacities: 396 days (3 topics)
- Expression of the DNA photolyases genes of *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) in DNA repair deficient human cells and to test its photoreaction activity
- Photoreactivation in human fibroblasts by baculovirus photolyases
- Identification of nuclear localization signals in PHR-2 from the baculovirus ChchNPV