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Papaver somniferum L. taxonomy, uses and new insight in poppy alkaloid pathways

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Abstract Since ancient times, opium poppy (*Papaver somniferum* L.) is known for its medicinal properties, related to its secondary metabolite content. Its most important secondary metabolites, called benzylisoquinoline alkaloids (BIAs), are still essential in pharmaceutical field. Few of them, like morphine, have specific clinical application but also effects on CNS. Not all poppy cultivars are able to biosynthesize morphine in high amount, making this plant useful for other purposes like food uses. For this reason it is crucial to deeply understand the origin of poppy, its possible use and have a deep knowledge of the BIA biosynthesis. These aspects are crucial for the final use of *P. somniferum*. This review aims to summarize the state-of-the-art on its taxonomy and origin beside its uses and BIA biosynthetic pathways, its most important metabolites. The review focuses on conflicting or unsolved questions about enzymatic localization, role of different plant organs in the biosynthesis, and storage and external conditions that influence the alkaloid production, highlighting the significant involvement of transcription factors. Behind this review, there is the firm belief that only a deep knowledge of alkaloid biosynthetic processes could lead to the characterization of undefined step and to the development of engineering cultivars optimizing the potential uses of *P. somniferum*. The goal is answer in more sustainable way to ever-increasing worldwide request of such products, in particular morphine and derivates, obtaining high morphine content cultivars useful for pharmaceutical market or no morphine producing cultivars appreciated as food. Devising cultivars with different BIA content could lead to decrease, or even avoid, illicit use and illegal extraction, confining only low alkaloid content cultivars to consumers market.

Keywords Benzylisoquinoline alkaloid · BIAs · Biosynthesis · Poppy · Phylogenetic · Transcription factors

Abbreviations

3′OHase	3′-Hydroxylase
3′OMT	3′-*O*-methyltransferase
3OHase	Tyrosine/tyramine 3-hydroxylase
4′OMT	3′-hydroxyl-*N*-methylcoclaurine 4′-*O*-methyltransferase
4HPAA	4 Hydroxyphenylacetic acid
4HPPDC	4-Hydroxyphenylpuruvate decarboxylase
6OMT	Norcoclaurine 6-*O*-methyltransferase

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7OMT	Reticuline 7-*O*-methyltransferase
AC	Adenylyl cyclase
AFLP	Amplified fragment length polymorphism
AMP	Adenosine monophosphate
AMPc	Adenosine monophosphate cyclic
BBE	Berberine bridge enzyme
BIAs	Benzylisoquinoline alkaloids
CAS	Canadine synthase
CFS	Cheilanthifoline synthase
CNMT	Coclaurine *N*-methyltransferase
CNS	Central nervous system
CODM	Codeine *O*-demethylase
CoOMT	Columbamine *O*-methyltransferase
COR	Codeinone reductase
CPVO	Community Plant Variety Office
CYP	Cytochrome P450 enzyme family
CYP82Y1	*N*-methylcanadine 1-hydroxylase
CYP80B	(*S*)-*N*-methylcoclaurine 3′-hydroxylase isozyme
Cys	Cysteine
DA	Dopamine
DBOX	Dihydrosanguinarine oxidase
EFSA	European Food Safety Authority
ER	Endoplasmic reticulum
EST	Expressed sequence tag
FT–ICR–MS	Fourier-transform–ion-cyclotron resonance–mass spectrometry
GC	Gas chromatography
GPCR	G protein–coupled receptors
HPLC	High performance liquid chromatography
MeJa	Methyl jasmonate
MIA	Monoterpenoid indole alkaloid
MLP15	Major latex proteins
MSH	*N*-methylstylopine 14-hydroxylase
N7OMT	Norreticuline 7-*O*-methyltransferase
NAc	Nucleus accumbens
NCS	Norcoclaurine synthase
NMCH	*N*-methylcoclaurine 3′-hydroxylase
NOS	Noscapine synthase
Nuclear factor-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OR receptors	Receptors for endogenous opiates
ORL1	Opioid receptor-like
P6H	Protopine 6-hydroxylase
PKA	Protein kinase A
PKC	Protein kinase C
PPH	Protopine-6-hydroxylase
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
RSP	Restriction site polymorphism
RVM	Rostral ventromedial medulla
SalAT	Salutaridinol 7-*O*-acetyltransferase
SalR	Salutaridine reductase
SanR	Sanguinarine reductase
SAT	(7*S*)-salutaridinol 7-*O*-acetyltransferase
SNPs	Single nucleotide polymorphism
SOMT1	Scoulerine 9-*O*-methyltransferase
SOR	7-Oxidoreductase
SPS	Stylopine synthase
STOX	(*S*)-tetrahydroxyprotoberberineoxidase
STRs	Short tandem repeat
STS	Salutaridine synthase
StySyn	Sstylopine synthase
T6ODM	Thebaine 6-*O*-demethylase
TFBSs	Transcription factor binding bites
TFs	Transcription factors
TNMT	Tetrahydroprotoberberine *N*-methyltransferase
TYDC	Tyrosine/DOPA decarboxylase
UHPLC–ESI–QTOF–MS	Ultra high performance liquid chromatography–electrospray ionization–quadrupole time-of-flight–mass spectrometry
VNTRs	Variable number of tandem repeat or minisatellites

Introduction

Papaver somniferum L. probably evolved from a wild Asian species, and it is one of the most economically important plant species widespread in temperate and subtropical areas of the hemispheres (Egan et al.

2011). Most of poppy uses are related to the secondary metabolites, in particular benzylisoquinoline alkaloids (BIAs) (Jablonická et al. 2016). BIAs are a group of eterogenous compounds, which shows a common biosynthetic origin and the importance of their accumulation results crucial for the final application of poppy. In fact they have a great importance in pharmaceutical market and research about these products is continuously carried on (Beaudoin and Facchini 2014). From this point of view, opium poppy has emerged as a model system to investigate BIA biosynthesis. Several reviews described important aspect of this specie, like the state of the art of morphine synthesis and biosynthesis, but there is still need for an analysis of its taxonomy and use(Beaudoin and Facchini 2014; Kutchan et al. 2004; Li and Zhang 2017; Michael et al. 2008). Alkaloid production is different in various cultivars, in this review there is a clear effort to summarize BIAs content for each cultivars, in order to pursue the main aim to identify selective markers to differentiate among cultivars. There is the firm belief that only a deep knowledge of alkaloid biosynthetic processes could lead to the characterization of undefined step and to the development of engineered cultivars. The goal is answer in more sustainable way to ever-increasing worldwide request of such products, in particular morphine and derivates, building high morphine content cultivars. Devising cultivars with different alkaloid content could lead to decrease, or even avoid, illicit use and illegal extraction, confining only low alkaloid content cultivars to consumers food market.

Origin and distribution

The genus *Papaver* belongs to *Plantae* kingdom, *Angiosperms* clade, *Ranunculales* order, *Papaveraceae* family and *Papaveroideae* subfamily. *Papaveraceae* is a cosmopolitan family of about 40 genera and 800 species, that grows from tropical to alpine ecosystems (Egan et al. 2011). The word poppy has been used for many species of the *Papaveraceae* family, while opium word has been used for the air dried latex extraction obtained from *Papaver somniferum L.,* one of the most useful plant species belonging to this family. All parts, but seeds, of plants of this genus are characterized by the watery and milky latex of vessels. About 240 species of the family, 170 belong to *Papaveroideae*, and the genus *Papaver* includes approximately 100 species (Fig. 1). At the tribal level, *Romneyeae* (two genera) have been separated from *Papaverae*, because of the different water-like colorless juice instead a milky latex that characterize them respectively. Species of genus *Papaver* are classified 9 sections in accordance with life cycle and related characters. Opium poppy and 3 other species (*P. glaucum*, *P. gracile*, *P. decaisnei*) form the most developed annual *Papaver* section (Tétényi 1997). An intraspecific classification of *P. somniferum* was proposed by Danert (1958). Danert (1958) proposed to differentiate *P. somniferum* in 3 subspecies [*setigerum* (DC.) Corb., *somniferum*, and *songaricum* Basil.], describing 52 botanical varieties. Four covariates (*alefeldii* K. Hammer, *somniferum*, *orientale* Danert, and *rothmaleri* K. Hammer) have been later described by Hammer (1981). Kadereit (1988) divided *P. somniferum* in two subspecies named: *setigerum*, and *somniferum*, where the first has been supposed to be the wild relative of the cultivated subsp. *somniferum*. Danert (1958) and Hammer (1981) based their classification on a few morphological characters like: capsule dehiscence, shape of stigmatic lobes, color of flowers and seeds. Nevertheless, the application of these characters is associated with some problems. In fact, it is not so easy to distinguish between round and angular stigmatic lobes and different seed colors may be present in the capsules of one plant (Dittbrenner et al. 2008).

The analysis of anthocyanin pigments (cyanidin and pelargonidin derivatives and in addition yellow compounds which were not anthocyanin) supports the proposed division of this genus into sections. Species of the same section develop the same anthocyanins, like pelargonidin and cyaniding (Acheson et al. 1956, 2006).

The reduction of lifetime and the development of anthocyanins are clear signals of differentiation due to evolution in the *Papaver* genus and they depend on more stressful condition of the late Industrial Age. Thus, we assist to a shift from perennial plant with yellow anthers to biennal or annual poppies with anthocyanated anthers (Tétényi 1997).

Given the importance of taxonomy, many attempts have been made to associate taxonomic proximity, to morphological similarity. Some studies showed seed character variations are useful for the taxonomy characterization at the specific and generic level,

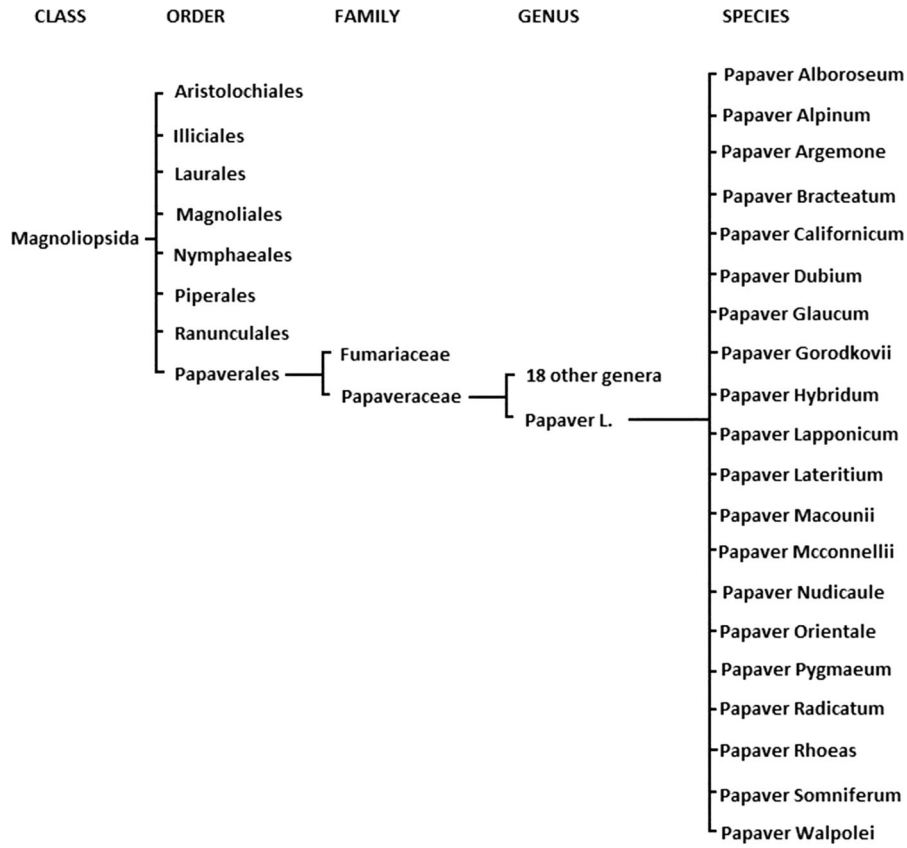

Fig. 1 Papaver spp. philogenetic tree

while, the morphology of leaf epidermis, with few exceptions, is one of the most useful character separation of taxa within species. Epidermal cell shape the anticlinal wall pattern, the testa sculpturing and the brightness of the seeds are he most taxonomically valuable characters related to *Papaver s*eed morphology, while ornamentation, shape and size, together with color of seeds are not reliable. Leaf epidermal morphology, due the similarity between species and the high variability among different population of same taxon, does not provide univocal characters for *Papaver* classification (Tavakkoli and Assadi 2016). *Papaver somniferum* probably evolved from a wild Asian species, or from a species called *Papaver setigerum*, which grew around the Mediterranean sea. Nowadays the cultivation of this species is widespread in both subtropical and temperate zones despite its natural habitat that is reported to be around the Mediterranean sea (Duarte 2005). Ethiopia, Scandinavia, Thailand, Tasmania and Argentina are examples of the different ecosystems that can be used for *Papaverum* species due to its high accommodative capability. The world opium poppy cultivation covers 270.000–300.000 ha. The 5 largest producers are India, Burma (Myanmar), Afghanistan, Turkey and the former Soviet Union, representing two-thirds of all cultivated fields. Among them India produces for world's pharmaceutical industries about the half of opium, from which it is possible to isolate codeine, morphine, narcotine, thebaine, papaverine and also produce other medical products (Tétényi 1997).

The poppy, in general, does not require any particular caution until the time of flowering; actually, its critical time is at the harvest, in July, because if it is not done at the right time, it could affect the quality of the latex produced by the poppy. The latex, which contains opium, could be collected only after the poppy has bloomed, the morphine level/content will be low if it is harvested too early, while the morphine will be biodegraded to codeine if it is harvested too late. Opium contains many natural alkaloids like narcotine, papaverine, codeine, thebaine, morphine (the most important) and some semi-synthetic products have been obtained starting from them (Froede

1972; Dang et al. 2012). Some studies tried to find a correlation between morphological characters and alkaloid content. An investigation of 210 opium poppy lines, drove the conclusion that large size of capsules and peduncles, small plant height, absence of pigmentation in flowers and low level of seed production are positively related to morphine high content. However, these correlation data showed correlation coefficient with values lower than 0.500. Furthermore it was showed that plant dry weight, capsule number and stigmatic rays are significantly correlated with opium, morphine and seed yield (Bajpai et al. 2000). Many other studies, instead, showed that it was not possible to detect strong correlation between morphological characters and major alkaloid content (Dittbrenner et al. 2008), because of high variability due to weather conditions. Hofman and Menary (1985) demonstrated as fungal activity, leaching and the changed activity of capsule enzymes could cause lower alkaloid content during wet weather; while higher temperatures as well as light intensity influenced positively BIA biosynthesis.

An investigation, carried out using twenty opium genotypes, showed a correlation between morphological traits and genotype. In fact, an association has emerged between fourteen characters and their direct and indirect effect on seed yield and latex yield independently. It revealed, from the path analysis for seed yield per plant, that number of effective capsules per plant exhibited maximum positive direct effect on seed yield followed by stem diameter, peduncle length, plant height and morphine content. Number of effective capsules per plant also exhibited a reasonable higher indirect effect on seed yield via seed oil content, days to 50% flowering, husk yield per plant, diameter of main capsule, morphine content and number of leaves per plant (Solanki et al. 2017).

This recent study has managed to overcome the limits of previous studies about variability, due to weather conditions. The morphological character observation is not the best method that could be used for the precise BIA content prevision. The BIA final amount can still be different in a single alkaloid or total alkaloid contents, due to highly variability of amount and composition of the main alkaloids in opium poppy, that definitely affect its final use.

Main uses of Poppy

Several part or product of this species have been investigated for their biological activities (Eryilmaz et al. 2016; El Nehir and Karakaya 2004; Prescha et al. 2014) but the commercial importance of opium poppy derives mostly from its medical properties, which are due to the production of several BIAs, that comprise about 2500 various structures. Several isolated BIAs display effective pharmacological such as: the narcotic analgesics codeine and morphine, the vasodilator papaverine, the cough suppressant and potential anticancer drug noscapine, the antimicrobial agents sanguinarine, cholesterol-lowering berberine and the muscle relaxant (+)-tubocurarine; most of them have been isolated from *Papaver* species, especially *P. somniferum* as a commercial source.

Poppy seeds are also appreciated in culinary field, in bakery production and for oil, and as an ornamental species. Opium poppy is one of the most valuable houseplant species. This study underlies as, nowadays, opium poppy remains the most important commercial natural source of drugs such as codeine, morphine beside a variety of semi-synthetic products, including oxycodone and buprenorphine, mainly derived from thebaine (Dang et al. 2012). Poppy genotypes are generally classified in three categories: industrial, when grown for alkaloid extraction from capsule; culinary when it is grown for seeds and oil production; both industrial and culinary when capsules and seeds are used for alkaloid extraction as well as seeds collection (Fig. 2). Another dimension of utility of the opium poppy plant is added by its ornamental usage in some countries (Singh et al. 2014). According to the area in which it is grown, the operational procedures of poppy cultivation change; for example in the areas like Western Europe and Australia where there is a high development of industrial production of poppy mainly for alkaloids, patented genotypes are used without cultivar registration by varietal authority. Because of their industrial value, details about origin and selection criteria of such genotypes are not provided. Instead, in other regions like Central Europe, cultivars are registered by appropriate cultivar offices where promising ones may be patented too.

The registered poppy cultivars in the European list maintained by the Community Plant Variety Office (CPVO) are differentiated based on their usage—industrial ('A1', 'Alfa', 'Botond', 'Evelin', 'Buddha',

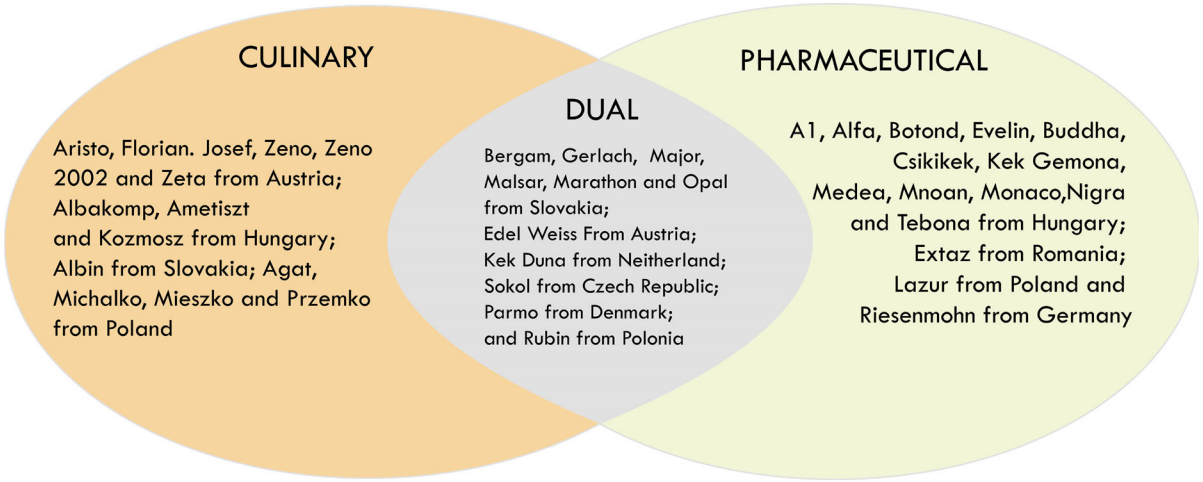

Fig. 2 Venn diagram for poppy cultivars' purposes and uses

'Csiki kek', 'Kek Gemona', 'Medea', 'Minoan', 'Monaco', 'Nigra', and 'Tebona' from Hungary; 'Extaz' from Romania; 'Lazur' from Poland; and 'Riesenmohn' from Germany), culinary ('Aristo', 'Florian', 'Josef', 'Zeno', 'Zeno 2002', and 'Zeta' from Austria; 'Albakomp', 'Ametiszt', and 'Kozmosz' from Hungary; 'Albin' from Slovakia; 'Agat', 'Michalko', 'Mieszko', and 'Przemko' from Poland) or dual ('Bergam', 'Gerlach', 'Major', 'Malsar', 'Marathon', and 'Opal' from Slovakia; 'Edel-Weiss' from Austria; 'Kek Duna' from Hungary; 'Marianne' and 'Rosemarie' from Netherland; 'Sokol' from Czech Republic; 'Parmo' from Denmark; and 'Rubin' from Poland). A third dimension of utility of the opium poppy plant is added by its ornamental usage in some countries (Singh et al. 2014).

The most important application is the use of poppy and its secondary metabolites is in the pharmacological field. In scientific terminology it is useful to distinguish between opiate and opioid terms. The first was used to designate the substances found in the poppy, but later came to include all compounds directly derived or synthesized from morphine and its derivatives. Thus, morphine, heroin, codeine and naloxone are considered to be correctly opioids, but fully synthetic compounds such as fentanyl are not.

Opioids refer to all substances, endogenous (natural binders for opioid receptors) and exogenous that interact with opioid receptors. Opioids are also known as narcotic analgesics, but often the definition of narcotic (a sleep-inducing drug) is used as a synonym for abused drugs (Kreek 2007). Opium contains about 25 alkaloids, including morphine, codeine and thebaine. Some of them together with some derivatives act on the endogenous opioid receptor system. The receptor subtypes designated as mu (μ), delta (δ), kappa (k) and opioid receptors (ORL-1) receptors are resumed in Table 1. Of these receptors, present in all vertebrates, there is ample evidence in the mammalian system; they are highly distributed in the central nervous system, including the brain and spinal cord, but also in the gastrointestinal system and in the immune system (Moloney 2016; Stefano et al. 2017).

For the reasons described above it is crucial to have access to an easy and rapid method able to distinguish between poppy varieties for food or pharmaceutical use, in order to reduce accidental consumption of morphine and, mostly, illicit extraction of morphine for heroin production from common commercial poppy seeds (European Food Safety Authority-EFSA 2011). An underestimated amount of illicit poppy use derives from ornamental poppy varieties. Few publications about the content of morphine in *P. somniferum* grown in gardens for ornamental purposes are available, even if the content of morphine in ornamental poppies cultivated in Estonia is not especially high, but absolutely remarkable from the point of view of potential use for narcotic purposes. It has been also indicated that ornamental poppies grown in home gardens could possibly be abused (Meos et al. 2017).

The complete understanding of biosynthetic pathway is necessary in order to develop or individuate

Table 1 Differential distribution of different OR types and related functions

Receptor type	Localization	Effects
μ_2, δ_2, κ_1 μ_1, κ_1, δ_1, δ_2	Laminae I and II of spinal cord (substantia gelatinosa). Periaqueductal gray, thalamic medial nuclei, thalamic intralaminar nuclei, raphe nuclei	Spinal and supraspinal analgesia
μ	Solitary tract nucleus, commissural nucleus, ambiguus nucleus, locus coeruleus, ipothalamic nuclei	Cough suppression
μ_2	Reticular substance, solitary tract nucleus, dorsal motor nucleus of vagus	Respiratory depression
μ, δ	Area postrema (CTZ), Edinger Westphal's nucleus, pretectal area, superior colliculus	Nausea and vomit
μ, δ		Miosis
μ	Hypothalam, posterior hypophysis	Inhibition of vasopressin secretion
μ, δ	Hypothalamic infundibulum, median eminence, accessory optic system, amygdala	Endocrinous effects
μ, κ, δ	Amygdala, hippocampal system, cortex, medial thalam, VTA, nucleus accumbens	Memory and behavior
δ		Motility
μ_2	Nucleus accumbens	Catalepsy and akinesia
μ	Caudate nucleus	Muscle stiffness
ORL-1	Septum, diagonal band of Broca, hypothalamus, hippocampus, medial amygdala, substantia nigra, locus coeruleus, raphe nuclei, PAG, dorsal and ventral horns of the spinal cord, trigeminal nucleus	Pain modulation

rapidly new poppy genotypes with low morphine content in latex or low latex production. Candidate genes, for this aim, are localized at first biosynthetic steps, to arrest the metabolic flow at first stages, when only precursors have formed and more competence is required to obtain drug from them. Another challenge would be try to obtain transgenic plants that can produce alkaloids in their organs and from were extraction is not so easy (Singh et al. 2014).

Biosynthesis

Opium poppy has emerged as one of the premier organism for the understanding of alkaloids metabolism. Over the last years, molecular studies about its genomic and transcriptomic have shown as this organism is a model in discovery of significant steps of biosynthesis of valuable pharmaceutical compounds. The presence of five chiral centers in the scaffold of morphine makes the chemical synthesis expensive, although possible. Thus, cultivation of opium poppy remains the only source of pharmaceutical opiates. All enzymes involved in the biosynthesis of some alkaloids, like sanguinarine, scoulerine and laudanine have been well characterized, as well as most of enzymes of morphinan pathway, except for reticuline epimerase (Beaudoin and Facchini 2014). Noscapine and papaverine pathways are not completely defined (Fig. 3). The combination of proteomic and genomic approaches lead to different results in content of BIAs in different parts of this species that suggest a different distribution of alkaloid biosynthesis in various parts of plant. The evidence of inability of dedifferentiated opium poppy cells in morphine production (Facchini 2001) suggests that morphine biosynthesis required specific cells type and that the expression of biosynthetic genes is related to their localization. The full comprehension of that can allow us to manage these genes to obtain cultivars using genetic engineering.

Morphine is extracted from latex of unripe capsules through manual lancing. This exudate, with complex chemical composition, immediately changes its color

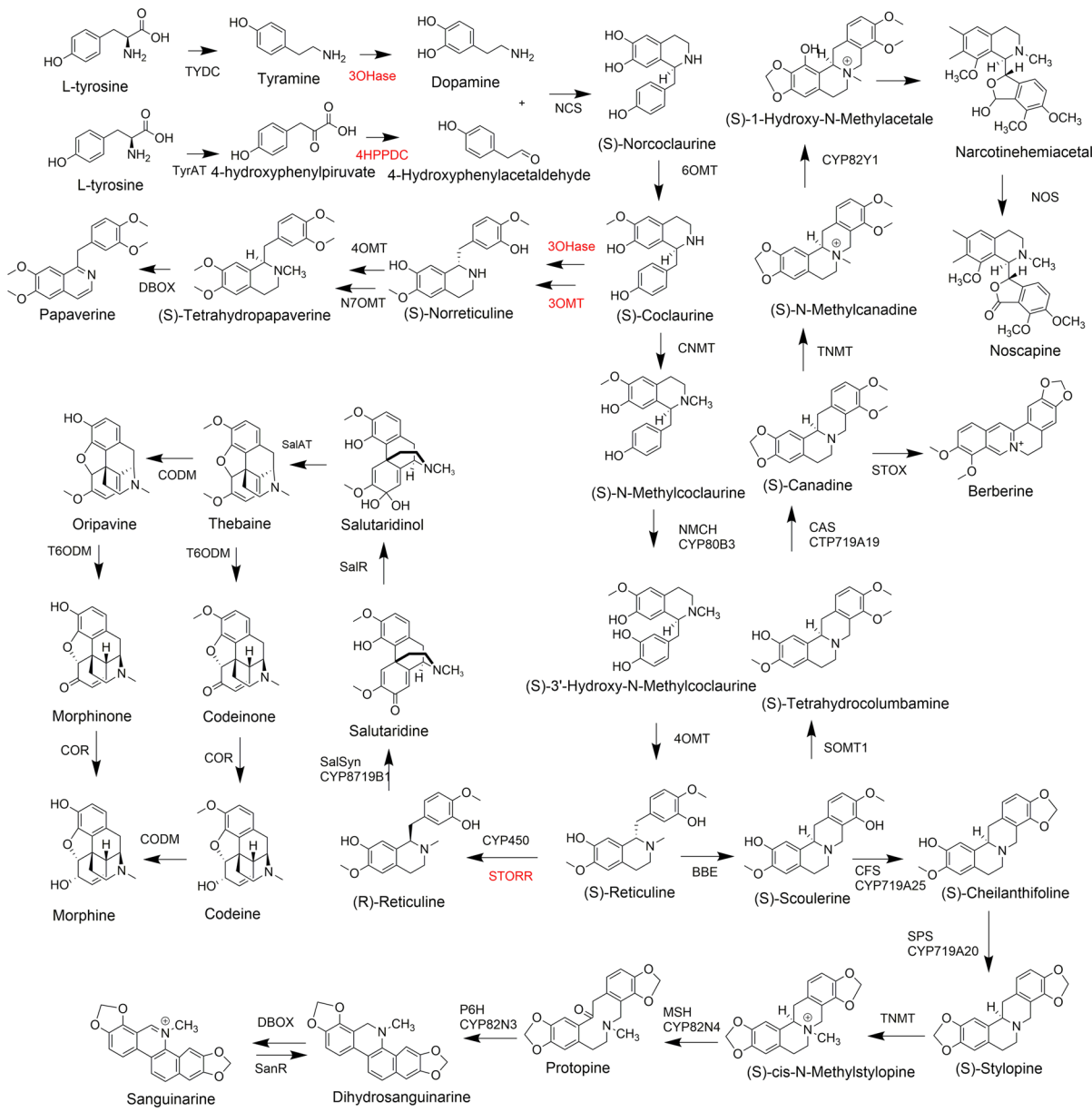

Fig. 3 Alkaloid biosynthetic pathway. Gene transcripts for enzymes labeled in red are not jet isolated and characterized

from white to brown after phenol oxidation by air exposure. It is the cytoplasm of laticifers, specialized secretory cells associated with phloem of plant. Phloem is a conduction tissue of lymph and it is formed by sieve elements, that could be distinguished in sieve cells and sieve tubes, the last one associated with companion cells, specialized parenchyma cells helping sieve tubes in their conduction role. Parenchyma cells surround laticifers within the large- and small-vascular bundle. All of them are visible in longitudinal section of stem. The poppy straw, which is the dried mature plant, also accumulates opiate alkaloids. Immunotechniques are powerful tools to understand specific localization of genes and enzymes. Kutchan et al. (2004) developed polyclonal antibodies targeted against 3′-hydroxyl-*N*-methylcoclaurine 4′-

O-methyltransferase (4′OMT), Salutaridinol 7-*O*-acetyltransferase (*Sal*AT), Reticuline 7-*O*-methyltransferase (7OMT), Codeinone reductase (COR), berberine bridge enzyme (BBE), and major latex protein (MLP) 15 that were initially evaluated by hybridization to protein gel blots that contained crude protein extracts from *P. somniferum* capsule, stem, leaf, and root.

Capsule

The major part of biosynthetic enzymes and MLP15 (a family of highly conserved, low molecular weight polypeptides typically expressed in laticifers) was found in capsule tissue and, with minor extent, in stem tissue, while BBE in stem and root. *Sal*AT and 4'OMT were detected in parenchyma tissue, likewise 7OMT, but only to parenchymal cells distal to laticifers. In contrast COR was found in laticifers, and only a small amount was located in parenchyma.

Stem

Like in capsule, cells near laticifers contained 4'OMT and *Sal*AT, while COR was located only in laticifers. The presence of 7OMT has been observed in vascular bundle but not in laticifers or sieve elements.

Root

Laticifers were not found in root tip, consequently COR was not immunologically detected. 7OMT and *Sal*AT were located in the pericycle of the stele, whereas BBE in root cortex (Kutchan et al. 2004). The occurrence of sanguinarine in roots implies that biosynthetic enzymes are restricted in roots. Anyway a possible answer of BBE mRNA in aerial organs could be in a translocation of sanguinarine biosynthetic intermediates from roots to aerial parts.

Norcoclaurine Synthase (NCS) is also most active in stems and roots while abundance of salutaridine synthase (STS) and 7-oxidoreductase (SOR) occurs in roots and shoots (Facchini 2001).

According to proteomic study, also transcriptomic data confirm this distribution: Tyrosine/DOPA Decarboxylase (*Tydc*) was largely reported in phloem of aerial plant parts; transcripts of 7OMT in phloem, and COR transcript only to laticifer cells. This hypothesis that only two type of cells are involved in alkaloid biosynthesis, parenchyma cells surrounding laticifers for early stages of morphine biosynthesis and laticifers for late stages and storage (Kutchan et al. 2004) has been broadened by Bird et al. (2003) that have established the role of sieve elements and companion cells. The first have proved to be the location of enzyme, while gene transcripts were found in the second (Bird et al. 2003). Cytoplasmatic vescicles of laticifers have an important role in storage. Until now any pathway have never involved sieve elements, it seems to be distinctive of BIAs and open new perspectives on vascular bundle system role. This distribution reflects the morphology of these two cell types: sieve elements are unable to make protein synthesis, due to the absence of necessary organelles, thus they depend by companion cell for enzyme synthesis. The limits of these studies, deriving from their focus on three of all enzymes and transcripts of complex biosynthetic pathway, have been overcome by Facchini and De Luca (2008), that observed seven biosynthetic enzymes: Norcoclaurine 6-*O*-methyltransferase (6OMT), Coclaurine *N*-methyltransferase (CNMT), (*S*)-*N*-methylcoclaurine 3′-hydroxylase isozyme 1 (CYP80B1), 4′OMT, BBE, *Sal*AT and COR localized to sieve elements in opium poppy and their corresponding gene transcripts to associated companion cells and confirm the absence of transcripts in laticifers (Facchini and De Luca 2008). This results allow to label laticifers as storage elements.

In an attempt to determine the cellular localization of BIA biosynthesis in opium poppy, the critical point is the localization of COR It has been shown a discrepancy between the two above mentioned studies, with one suggesting its occurrence in laticifers (Kutchan et al. 2004) and the other its exclusive association with sieve elements (Bird et al. 2003). As such, the cell type–specific localization of morphine biosynthesis in opium poppy remains controversial.

An attempt to reconcile these apparently disparate results was made by Onoyovwe et al. (2013), that evaluated the localization of all six biosynthetic enzymes of the morphine pathway in *P. Somniferum* in parenchyma cells and sieve elements. Sieve elements have distinctive morphological traits, like sieve plates that could be blocked by a substance called callose, they and also express a special protein called slime body and an H^+-ATPase isoform that

make them identifiable. The conflict is also about latex role. The presence of some transcripts, mainly COR transcripts, in latex could be waver the thesis of laticifers as storage elements. Although immunolocalization has proven to be an useful for the detection of BIA biosynthetic enzymes in sieve elements, the unique nature of the vesicle- and MLP-rich latex, which could mask proteins from immunological detection in fixed and resin-embedded tissues, makes it a not reliable method for protein localization in opium poppy laticifers. According to this theory, also in other study *Sal*AT and COR mRNA and corresponding enzymes in laticifers couldn't be detected, although transcripts have been reported in laticifers. Noscapine Synthase (NOS) and 7OMT have been detected in laticifers. These enzymes are involved in the final steps of the formation of noscapine and 7-*O*-methylated derivatives of reticuline. This results indicate that their synthesis is spatially separated from upstream enzymes (Beaudoin and Facchini 2014).

In conclusion, depending on the chemotype, morphinans have been revealed as the most abundant alkaloids in the latex following by noscapine and papaverine. In contrast, in roots the most abundant alkaloids are sanguinarine and dihydrosanguinarine. Opium poppy, as premium model organism, qualify as an interesting object of study to investigate BIA metabolism, given that all known enzymes involved in it are represented in this plant specie (Hagel and Facchini 2013).

This intricate network of transcription, tradition and storage requires an efficient transport system and generates doubts about what is the stage where the translocation occurs and what intermediates involves. Little is known about this, thebaine and narcotine hemiacetal seem to have a significant role in this sense, although the presence of almost all pathway intermediates, suggests the unspecificity of process. Recently, a multidrug-resistance-type, ATP binding cassette (ABC) protein from *Coptis japonica* (CjMDR1) was shown to transport the benzylisoquinoline alkaloid berberine (Beaudoin and Facchini 2014).

Major limitation to understanding BIAs biosynthesis is due to limited genomic information about opium poppy and relative inefficiency of genetic transformation protocols. Its genome's large size of about 7.4 Gb and relative inefficiency of genetic transformation protocols, makes the complete sequencing a hard challenge. Nevertheless, the recent high-throughput technologies such as genome sequencing, expressed sequence tag (EST) and DNA microarray analyses, proteomics and metabolomics have been shown useful tools to improve the knowledge of new genes involved in BIA metabolism.

Trafficking and subcellular compartmentalization

Due their toxicity, the new synthetized alkaloids and their intermediates have to be stored outside of cytosol. This trafficking constitutes an important checkpoint, thanks to close regulation that avoid the free diffusion. Investigations about localization of sanguinarine biosynthetic pathway, focused on five non-cytosolic enzymes that lead on its production, showed that they are associated to membrane with a density consistent with that of endoplasmic reticulum (ER). The necessity of being associated with ER depends from the nature of enzymes. Except for BBE, all of them are P-450 dependents, so they must be related to ER (Facchini 2001). The ER of sieve elements seems to be widely involved in BIA pathways; it is demonstrated by its extensive dilation following the induction of expression of BIA biosynthetic genes (Liscombe and Facchini 2008). Although BBE appears as a soluble protein, differently from other ER membrane bound enzymes, it is initially labeled with an *N*-terminal signal peptide of 25-amino acid to the ER to translocate to the vacuole. Acid pH conditions of vacuole is incongruent with optimal pH activity of BBE, thus it suggests that alkaloids synthesis occurs before the translocation and it is completely associated with ER. (*S*)-reticuline is imported from cytosol to ER in order to be converted in (*S*)-scoulerine, that is subsequently exported. The presence of sanguinarine in vacuole results from its translocation by transport vesicles to vacuole. In vacuole sanguinarine and related intermediates are not accumulated, but secreted and bounded to wall in order to be reabsorbed, decreased in toxicity and metabolized (Facchini and De Luca 2008). Also STS enzyme, which is involved in morphine biosynthesis, is associated with ER; likewise NCS, at the beginning of pathway. This enzyme shows the same complex trafficking of BBE. The compartmentalization of NCS in the ER lumen requires the concomitant translocation of dopamine and 4 Hydroxyphenylacetic acid (4HPAA) to ER. (*S*)-norcoclaurine is promptly exported for further reaction by *O*- and *N*-

methyltransferases cytosolic enzymes. Recently Cyp80B3, BBE and sanguinarine were co-localized with the ER in opium poppy cell cultures.

Transcriptomic, proteomic and metabolomic

Secondary metabolism in plant is prone to many external influences that overcomplicate its understanding (Mitrová et al. 2018; Fernández et al. 2013). To make light on production of secondary metabolites, an integrated approach between gene expression and metabolite profile was developed. In this way EST library and microarray analyses revealed to be useful technology to survey the transcriptome, while LC–MS/MS to and Fourier-transform ion–cyclotron resonance–mass spectrometry (FT–ICR–MS) profiled the proteome and metabolome, respectively. The correlation among specific alkaloids and the corresponding expressed sequence tags (ESTs) encoding known benzylisoquinoline alkaloid biosynthetic genes demonstrated the feasibility of bilaterally predicting enzyme function and species-dependent specialized metabolite profile, that could be used to characterize unknown gene and enzymatic pathway. The database containing full/length mRNAs transcripts encoding most alkaloid biosynthetic enzymes is an essential condition for the functional characterization of novel gene candidates (Hao et al. 2015). Deepen knowledge of the pathway that underlie BIAs biosynthesis should allow us to develop engineering crop for production of these important pharmaceutical molecules.

Papaverine, synthetized in low amount in most varieties of *P. somniferum* is known for its vasodilator, muscle relaxant and antispasmodic properties. Papaverine biosynthesis still remains controversial. In order to clarify this pathway, transcriptomic profile of mutant of opium poppy (Pap1) which accumulates papaverine up to 6% of the latex with visible phenotype of white latex in comparison to normal cultivar (BR086) with 0–0.5% papaverine of normal pink latex were carried on. Analysis of contigs led to the characterization of 4′OMT, 6′OMT, CNMT, 7OMT and Norreticuline 7-*O*-methyltransferase (N7OMT) with different expression between two mutants. As Pap1 produces higher amounts of papaverine, up regulated genes will be surely involved in papaverine biosynthesis (Fig. 4). Results showed up regulated levels of expression of 4′OMT, 6′OMT, CNMT and N7OMT in Pap1, while 7OMT was more than fourfold down regulated. 4′OMT is up regulated more than threefold in Pap1 in comparison to BR086. These evidences suggest that N–CH3 pathway that utilizes (*S*)-reticuline is not preferred way to synthesize papaverine, but it occurs through NH pathway, solving the controversial question of papaverine biosynthesis (Pathak et al. 2013).

Noscapine was first found in opium poppy, but in spite of its belonging to opiates, noscapine has not painkilling role and related addiction problems. It was used over the years as a cough-suppressant, but recently it has emerged for its anti-cancer effect, due to its ability in tubuline binding and apoptosis induction. Also this time, comparison between mutant species helped in elucidation of biosynthetic pathway. Analysis of gene expression and metabolite profile on high morphine-, high thebaine-, and high noscapine-mutants revealed previously unknown genes only in last mutants. Using HN1 and HM1 as parents, we generated an F2 mapping population of 271 individuals. Crossing experiments revealed that the HN1-specific genes are tightly linked and associated with the presence of noscapine, which suggests that they occur as a gene cluster involved in noscapine biosynthesis (Winzer et al. 2012). Besides being co-expressed, these genes are also co-regulated, as demonstrated by Kakeshpour et al. (2015) that found multiple unique transcription factor binding sites (TFBSs) of many known TFs in the promoter regions of these 10 co-expressed genes.

The same approach was used for morphine, whose extraction from poppy is essential, due to unfeasibility of its chemical synthesis. Thus, the knowledge about morphine biosynthesis could allow to manage morphine content to have functional cultivars that, from one side, could supply more amounts of morphine in pharmaceutical purpose cultivars, while on the other hand, it could avoid illicit usage or accidental contamination of food products from poppy. The poppy mutant known as thebaine oripavine poppy 1 (*top1*), which accumulates the morphine and codeine precursors thebaine and oripavine and does not complete their biosynthesis into morphine and codeine is a precious tool to investigate morphine pathway. It showed 10 down regulated genes. Out of these, 6-*O*-demethylase is the most important because is responsible of the conversion of thebain and oripavine, which

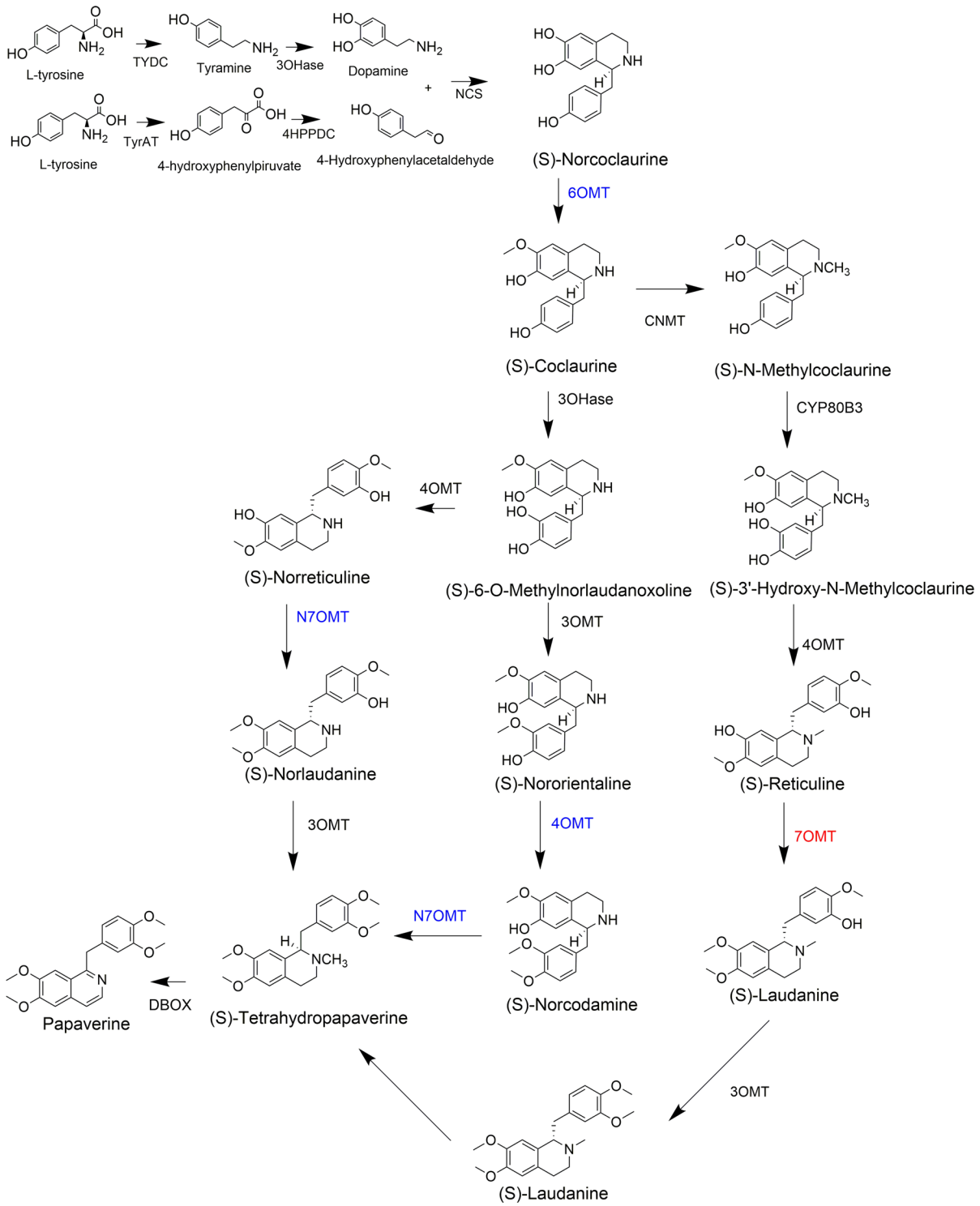

Fig. 4 The two proposed biosynthetic pathways for Papaverine. Enzymes in blue are over-regulated, while enzymes in red are down-regulated, leading to the conclusion that papaverine biosynthesis occurs through NH pathway (left) and not through NCH3 pathway (right)

is accumulated, in morphine. Reasons that could explain this stop, have been found in an alteration of transport mechanism or in a regulation by enhancers or repressors on operon site (Batterham et al. 2004).

Variability

The availability of the transcript, protein, and metabolite profile databases provides an overview of the great variability of BIA array in different cultivars of poppy. Such databases were builded comparing gene expression pattern with related BIAs accumulation in different species and organ or tissue to find best candidates. Paralogous and orthologous were found in other plant species to understand function and to detect responsible amminoacids of such function (Ziegler et al. 2009). Gene candidates are selected basing on phylogenetic similarity and correlations between metabolite and transcript abundance profiles (Desgagné-Penix et al. 2010). The gene expression profiles of *P. somniferum* in several *Papaver* species were compared with microarray technologies in different following studies. Even if such research is complicated by necessity to compare species that could not have same development or could show morphological disparity. In order to overcome those matters, many species were included and steams revealed as the best part of plant, on which growth conditions have less visible interfering effects. Ziegler et al. (2009) reported that overexpression of genes for morphinan pathway, STS, Salutaridine Reductase (SalR), SalAT, and CoR1, that acts downstream of *S*-Reticuline pathway, was shown only in *P. Somniferum* species, confirming this specie as main source of opiates. Remarkably, *P. bracteatum* and *P. arenarium* showed a morphinan compound in their profiles of alkaloid production correlated to a high relative gene expression. No substantial differences were found in expression of genes involved upstream of *S*-Ret pathway between species. Expression of BBE is related to noscapine production and high noscapine level species, but it is interestingly contrasting, because it also occurs in species that do not produce noscapine, and sometimes in low level in noscapine producing species. This result get off the direct correlation between BBE expression and noscapine production, in fact BBE is involved in the biosynthesis of more than a quarter of all known BIAs. Generally gene expression was found higher in *P. somniferum* related to other poppy cultivars and, except for morphinan pathway, it seems that there are not explanations for this. From analysis of such differences, novel cDNAs have been characterized: cDNA as a N7OMT. Abundance of N7OMT transcripts in high papaverine species suggested its involvement in papaverine biosynthesis; P450 enzyme as salutaridine synthase; cDNA correlated to an enzyme that catalyze opposite reaction of salutaridine reductase (Ziegler et al. 2009).

A second study, carried on 8 different cultivars, bring to broad databases and to clarify alkaloid distribution in different varieties. It was performed integrating analysis of transcript and metabolic data, using ESTs and HPLC, respectively. Collected data has shown in Fig. 5.

Desgagné-Penix et al. (2010) classified the various cultivars into 5 main groups on the occurrence of major alkaloids relative to other BIAs: (1) low overall alkaloid levels (Przemko), (2) high levels of thebaine and oripavine, and low levels of codeine and morphine (T), (3) high-codeine and morphine levels, but no other major alkaloids (40 and Deborah), (4) codeine and morphine accumulation along with high narcotoline and/or noscapine levels, but without papaverine (Marianne, Natasha), and (5) codeine and morphine accumulation together with high narcotoline/noscapine and papaverine levels (Roxanne and Veronica).

There is no univocal correlation between transcript encoding enzymes in several pathways and BIA production, many pathways leading to morphine have been found in high Pap varieties.

Accordingly, Desgagné-Penix et al. (2012) lead to a characterization of novel gene candidates encoding CYP family members involved in papaverine biosynthesis. Such CYP has shown high similarity with NMCH that catalyzed a hydroxylation reaction, like that occur on (*S*)-Coclaurine during biosynthetic steps that lead to papaverine.

Composition of the main alkaloids was evaluated also in 115 Indian landraces of opium poppy (Prajapati et al. 2002). Of all 115 accessions, 71 accumulated all alkaloids, while 36 were papaverine deficient, 7 papaverine and narcotine deficient and 1 morphine and codeine deficient. In the 71 accessions that accumulate all alkaloids, morphine occurs as major constituent followed by codeine, narcotine, papaverine and thebaine. Among the intermediates, the amount of codeinone was relatively more as compared to reticuline and oripavine. Accessions harboring one

or more blocks, as commonly happens in wild races, seem to have less alkaloid content rather than normal cultivars. Low papaverine content cultivars showed the same alkaloid content, this confirms the involvement of nor-reticuline way in papaverine biosynthesis, without influencing reticuline pathway alkaloids. Low morphine/codeine content cultivars contain a higher amount of papaverine, reticuline, narcotine and codeinone. As oripavine level remains more or less the same and codeinone got accumulated, it seems that morphine is mainly synthesized by codeinone. Thebain content was significantly correlated to codeine content, which was highly correlated to morphine content. The amount of reticuline was significantly correlated to that of codeinone, but negatively to that of codein, demonstrating that probably codeinone reductase was rate limiting. Narcotine content had significant positive correlation with morphine, papaverine and reticuline. Thus when the synthesis of reticuline was augmented, the profile of morphine, codeine, thebaine and level of narcotine also got increased. Reticuline, however, did not seem to go preferentially towards narcotine, as the levels of codeinone and papaverine also got augmented (Prajapati et al. 2002). The confirmation that morphine is the main alkaloid in nearly all accessions, while the composition in other main alkaloids is high variable comes from Dittbrenner et al. (2008) that evaluated about 300 accessions concluding that any attempt to classifying poppy species basing on alkaloid content is not reliable, due to the so many conditions (weather, growth stage) that can influence plants. The distinction between all cultivars by unique characters is important to have a whole overview of genetic relatedness (Ovesná et al. 2013). RAPD and AFLP demonstrated a powerful techniques to differentiate among the species, as shown by Darokar et al. (2014) that characterized 5 *P. somniferum* cultivars (Sampada, Sujata, Shyama, Sanchita and Shweta) and found that all of them fall within the similarity range of 85–100%. The building of a similarity dendrogram, showed that opium poppy parental lines could discriminate univocally *P. somniferum* cultivars among the others (Saunders et al. 2000).

The necessity to individuate differences among poppy accessions and building a genetic mapping of opium poppy results in unstoppable research of markers in polymorphic sites. Single Nucleotide Polymorphism (SNPs), Restriction Site Polymorphism (RSP), Restriction Fragment Length Polymorphism (RFLP), Variable Number of Tandem Repeat (VNTRs) or minisatellites, Short Tandem Repeat (STRs) or microsatellites are the main variation that could be found in plant genome. The use of microarray or restriction enzyme, as well as sequencing is a useful way to detect them. Despite its clear importance, also related to great help in selection of best crops for different aims, only few studies were conducted about. A breakthrough in this sense was made by Şelale et al. (2013) that increased the number of EST-SSR markers for Papaver by tenfold. These results were overcame by Celik et al. 2014) that developed genomic SSR markers in opium poppy, using high-throughput pyrosequencing and the 160,000 obtained reads cover 105 Mb of the opium poppy genome, equal to 2.83% coverage of the entire genome, exceeding the 0.4% of previous study.

Signal transduction and inducible expression: role of transcription factors

Secondary metabolites have been often demonstrated to have an important role in plants defense by external injury, like weather conditions (drought and cold), fungal damage, wounding, UV rays, osmotic shock and heavy metals (Russo et al. 2015; Sofo et al. 2010). Obviously, in order to obtain more information about BIAs production, several variable conditions have been used to increase and study secondary metabolism. In opium poppy, the most BIAs enhancing factors could be found in elicitor, methyl jasmonate (MeJa) treatment and wounding. Concentration of elicitor that stimulate the BIAs production is lower than that induce production of phenolic compounds and hypersensitive response, a defense mechanism used by plant in order to reduce infection, which is set out through induction of death in cells surrounding injury site. It is activated by reactive oxygen species that seem to not be involved in phenolic production, because it is selectively blocked by catalase at higher elicitor concentration. The induction of benzophenanthridine alkaloid biosynthesis after elicitor treatment showed the need of a temporary acidification of cytosolic pH caused by an efflux of protons from the vacuole. Such acidification was reproduced in vitro and it revealed the possibility to induce alkaloid biosynthesis but not the hypersensitive response, while the increase of pH stopped BIAs production.

Efflux of vacuolar H^+ could be initiated by a phospholipase A2 G-protein (Facchini 2001). Phospholipase A2 (PLA2) in *Papaver somniferum* is responsible of lysophosphatidylcholine production and linoleic acid, with remarkable properties in plant defense, and generally, in cellular protection. Thus it seems reasonable admit that PsPLA2 and its reaction products are related to the production of opium poppy secondary metabolites with possible roles in defense reaction mechanism, such as synthesis of BIAs (Jablonická et al. 2016). The main difficult linked to research about inducible alkaloid pathways by different factors is the inability of cell culture to produce morphinans, differently from whole plants. Only sanguinarine and macarpine synthesis and storage after elicitor treatment is observed. In general, the membrane-associated biosynthetic enzymes are induced by elicitors, whereas most cytosolic enzymes are not (Liscombe and Facchini 2008). The most important TF families are MYB and WRKY, the first are the largest TFs superfamily in eukaryotes, while the second represent a superfamily of plant specific TFs. MYB is responsible of STR gene expression that is the main enzyme involved in Monoterpenoid indole alkaloid (MIA) biosynthesis. The hallmark of MYB family is an *N*-terminal MYB domain with imperfect tandem repeats, composed by a region of 50–53 amino acids containing three regularly spaced tryptophan/phenylalanine residues. Interaction with DNA are guaranteed by alpha-helix fold of each repeat and hydrophobic tryptophan core that allows DNA-proteins interplays (Jia et al. 2004; Inukai et al. 2017). WRKY is responsible of expression of genes involved in BIA production and generally in secondary metabolism, in response to various stresses. It is detectable in major amount in leaves of *P. somniferum*, that is an annual plant, and in roots of *P. bracteatum*, that is a perennial plant. This different localization confirms the important role of WRKY in plant growth and developmental processes like leaf senescence, seed maturation, embryogenesis, and germination. W-box, containing conserved consensus motif TGAC(C/T), is an important regulatory element sequence that is located in promoter sequence of stress-induced genes and can regulate gene expression through recognition by WRKYTFs. They contain a 60 residue domain with the conserved *N*-terminal WRKYGQK core sequence before a zinc finger motif (Wang et al. 2016). WRKY family protein was found to be encoded by PsWRKY, a sequence of 1797 bp expressed in *P. somniferum* upon stress treatment like wound (Mishra et al. 2013), MeJa, salt, cold and dry. PsWRKY protein has capacity to bind specifically to W-box, as demonstrated by gel retardation assay, which showed a shift for PsWRKY in presence of probe containing W-box sequences, while GST-fused antisense clone of PsWRKY did not, because of *N*-terminal domain is implied in GTS fusion (Mishra et al. 2013).

In *P. somniferum* there is a competition between BIAs and MIAs production. Acting on MYBTFs is a good strategy to decrease STR expression and related MIAs production, thereby facilitating BIAs production for further investigations (Kakeshpour et al. 2015). In order to identify the TF families and their members in different poppy cultivars, several studies were carried on, starting by sequences from plant TF databases compared with transcriptome data (contigs) collected from 10 different cultivars of poppy with different BIAs profile for homology search through BLAST database. Analysis results led to detection of genes encoding members of TF families, especially papaverine-specific TF. Several contigs showed significant up regulation in pap1 (High Pap Mutant) as compared to BR086 (wild type) and expression of contigs annotated as WRKY TFs was observed only papaverine producing cultivars. Furthermore, W-boxes were found in promoter region of these genes and others involved in BIAs pathway. Thus, such contigs could belong to TF family and have a significant role in regulation of papaverine gene expression and, in general, of alkaloids (Agarwal et al. 2016). More recently, Winzer et al. (2012) reported a gene cluster of 10 genes for noscapine biosynthesis. The presence of W-box in promoter region of this clusters highlights the important role of WRKY TFs in noscapine production and suggest that biosynthetic gene in noscapine pathway are co-expressed and co-regulated (Winzer et al. 2012). As reported above wounding and MeJa treatment are conditions that induce the production of BIAs. Monitoring the production of BIAs during elicitor or MeJa treatment could led us to a better characterization of biosynthetic pathways, because of consistent accumulation of BIA biosynthetic transcripts and related enzymes after elicitor or MeJa treatment in cell cultures or in the whole plant. Correlation of transcriptomic and proteomic analysis using sequencing

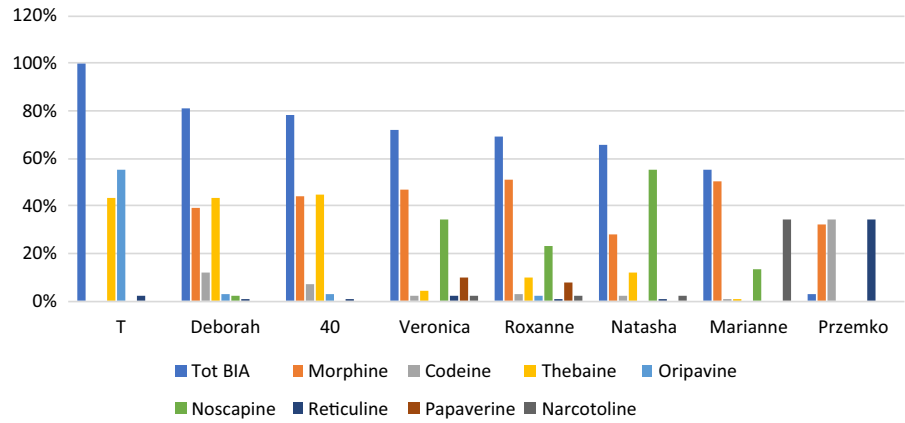

Fig. 5 Differential distribution of major benzylisoquinoline alkaloids (BIAs) in latex extract of 8 different *Papaver somniferum* cultivars

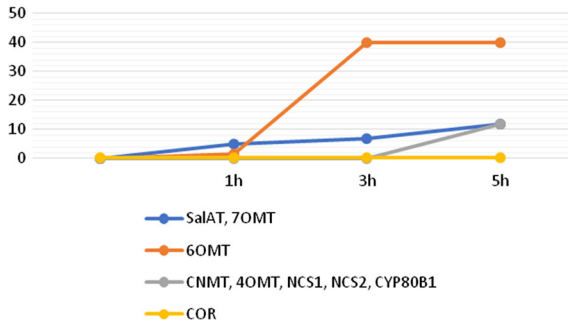

Fig. 6 Transcripts induction after 1, 3 and 5 h of wounding. After 1 h only SalAT and 7OMT transcripts appear overexpressed; after 3 h 6OMT transcript increases its expression of 40-fold, followed by SalAT and 7OMT; after 5 h all biosynthetic genes are induced more than tenfold. Only COR does not show changes in expression level

and chromatography are powerful tools to study natural product biosynthesis in plants and to try to delineate complete genome sequence. This bilateral analysis, at several times during induction, facilitates the correlation between occurrence of specific transcript and abundance of related alkaloids.

In particular it was demonstrated an induction after wounding of TYDC, CNMT, 6OMT, 4′OMT, BBE, and (7S)-salutaridinol 7-*O*-acetyltransferase (SAT) transcripts. A cDNA library, composed by 1500 recombinant clones was used for hybridization studies that led to isolation of 80 EST with twofold induction after 5 h of wounding. Comparison of transcriptomic and proteomic showed that transcripts for different biosynthetic enzymes were induced at different times. COR was not induced after wounding and consequently morphine levels in plant have showed a reduction. As result of over expression of other biosynthetic genes, narcotine and papaverine increased. While codeine and sanguinarine levels remained unvaried (Mishra et al. 2013). After MeJa treatment also COR showed tenfold induction after 3 h (Fig. 6).

From treatment of cell cultures with elicitor for 10–50 h it emerged that out of all overexpressed transcript, six belong to sanguinarine pathway: NCS, CNMT, BBE, stylopine synthase (StySyn) and two putative Tetrahydroprotoberberine *N*-methyltransferase (TNMT) isoforms. A rapid and transient expression of several members of the TYDC gene family was observed, levels of BBE were increased and CYP80B1 transcript levels were induced more than 20-fold in treated opium poppy cells. The elicitor-mediated induction of other P450-dependent enzymes Cheilanthifoline Synthase (CFS), Stylopine Synthase (SPS), *N*-methylstylopine 14-hydroxylase (MSH), and Protopine-6-hydroxylase (PPH) in the sanguinarine pathway has also been observed (Liscombe and Facchini 2008). As expected, no one of morphine pathway genes has been found, consistently with the claimed inability of dedifferentiated cells to produce morphine (Desgagné-Penix et al. 2010). On the contrary, in plant system, also morphinan pathway was expressed. In addition to NCS, CNMT and methyl-transferases, already found, also COR and SalR, typically expressed in morphine synthesis, showed altered levels of gene expression in poppy

capsule tissue. From proteomic analysis, all alkaloids showed an increase of production after 3 h that keep growing until 12 h of treatment, where morphine and noscapine reached the highest levels. However the levels of papaverine and laudanosine are low, about tenfold compared to other BIAs (Gurkok et al. 2015).

Due the complexity of metabolic pathway in poppy, and in general in plant, an approach aimed at engineering of TFs could be revealed more useful than many attempts to manipulate enzymes, also thanks to great progresses in discover of TFs binding sites and a better characterization of regulatory sites on promoter (Kakeshpour et al. 2015). Manipulation of TFs could allow us from one hand to overexpress genes involved in production of product of pharmaceutical interest, from the other to repress expression of those genes responsible of negative effects on plant, like production of compounds that lead plant or its fruits to root, and extend plant life with clear benefits in agronomical field. However, TF are not free of problems, In fact it is difficult to establish the exact function of each TF members due to their high similarity and unknown relationships between them. Also, to have a complete overview of TFs properties in the whole plant during all growth stages results is complicated because until now all conducted studies take in consideration only one stage of development in controlled environment. This condition is very different from cultivation adopted during breeding. Last but not least problem to be evaluated is the risk to obtain transgenic plants with other changes in their phenotype and genotype rather than that desired. Many progresses have been made but many have still to be done in order to clarify the intricate network between TFs and promoters of stress-inducible genes and discover new candidate genes sensible to stress that could be manipulated in order to address the appropriate use of *P. somniferum* genotype (Wang et al. 2016).

Conclusions

Biological processes related to biosynthesis and accumulation of most important BIAs in plants, especially in opium poppy, have been elucidated more and more over the years. On one side, lack of complete genome sequence has impeded the research in this field, but on the other the great collection of contigs, unigenes and genes together with advanced trascriptomic and proteomic tools have allowed to elucidate most of biosynthetic step in alkaloid pathway. As reported by Facchini (2001) "Plant alkaloid biosynthesis is more than a mere metabolic curiosity resulting in the formation of an immense array of biologically active products". Its regulation depends on several factors, like environmental stresses, lead to transcription of transcription factors, enhancers and repressor, and involves an intricate network of cells signaling. The elaborate subcellular compartmentation of enzymes and the intercellular translocation of pathway intermediates confirm the complexity of plant metabolism. Many issues are still to be solved, but the ever-growing availability of technologies and molecular tools opens up great horizons in identifying unknown pathways or other regulatory factors in alkaloid gene expression. Metabolic engineering with transgenic plants, heterologous system, new cloning vectors and new model organisms will also lead to concrete opportunities to clarify unresolved question, given the socioeconomic importance of poppy derivatives drugs. Seeing the increase of global demand, there is a strong necessity to develop specific engineering cultivars with high opium amounts, as long as plant extraction will remain the only source of these precious compounds.

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