Identification of Gene Involved in Cypress Canker by PCR-Select Subtractive Hybridisation Approach

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Abstract – Cypress canker is the most serious biological threat faced by cypress in Europe and North America. Tree breeding strategies retain canker resistance the primary selection criterion.
Identification of genes activated or inhibited during the infection process is the basis to better understand the canker resistance. PCR-select (suppression subtraction hybridization) technique of isolation of genes specific for an infection process, was applied for analysis of host-pathogen interactions in the pathosystem *Cupressus sempervirens / Seiridium cardinale*. The subtraction, with RNA from the early stages of infection of *S. cardinale*, as a tester, and RNA from uninfected *C. sempervirens*, as a driver, enriched the pool of cDNA molecules with the ones specific for infection.
The first step, was to develop a critical protocol for RNA isolation from cypress bark to provide a good quality of RNA for the further analysis. In a second step, 5 years-old seedlings of *C. sempervirens* were artificially infected by virulent strain of *S. cardinale*. Particular attention was paid in the experimental design to avoid to select genes that were activated only by wounding. A third step, was the isolation of pathogen DNA to monitor, by Real-time PCR, the pathogen spatial colonization in the bark along the stem. In the fourth step, a subtractive procedure to obtain an enriched library of cDNA, by PCR-Select, was carried out to select putative genes. To this purpose databank similarity searches were performed with the Blastx. program maintained at NCBI. In this study we succeeded in identifying about 100 cDNA clones significantly expressed in infected hosts but not in the uninfected control. The expression of several of these genes showing sequence similarity with resistance- or stress-related genes from other plant species were identified.

***Cupressus sempervirens* / *Seiridium cardinale* / gene expression / cDNA / fungal colonization profile**

Kivonat – A ciprusrákkal kapcsolatos gének azonosítása PCR alapú szubtraktív hibridizációs megközelítéssel. A ciprusrák Európában és Észak-Amerikában a ciprusok legjelentősebb biológiai veszélyeztető tényezője. A nemesítési stratégiák elsődleges szelekciós szempontja a rákkal szembeni ellenállóság. A rákkal szembeni ellenállóság jobb megértésének alapja a fertőzési folyamat során aktivált vagy gátolt gének azonosítása. A *Cupressus sempervirens / Seiridium cardinale* patoszisztéma gazda-kórokozó kölcsönhatásának elemzése céljából a fertőzési folyamatra specifikus géneket PCR alapú technikával izoláltuk. A szubtrakció, a *S. cardinale* fertőzés kezdeti szakaszából származó RNS teszterrel és a fertőzésmentes *C. sempervirens* RNS vezérlővel, megemelte a fertőzésre specifikus cDNS molekulák számát.

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Első lépésként kidolgoztuk az RNS kivonásának kritikus módszerét a ciprus kéregből, jó minőségű RNS nyerése céljából a későbbi vizsgálatokhoz. Második lépésben ötéves *C. sempervirens* csemetéket fertőztünk virulens *S. cardinale* törzzsel. Különös figyelmet fordítottunk a csak a sebzés által aktivált gének kiválasztásának elkerülésére. A harmadik lépés a kórokozó DNS-ének izolálása volt, Real-Time PCR módszerrel, a kéreg kórokozó általi térbeli kolonizációjának monitorozása céljából a törzs hosszában. Negyedik lépésben, a cDNS könyvtár bővítésére, PCR alapú szubtraktív eljárást folytattunk a szóba jöhető gének kiválasztására. E célból hasonlóság keresést végeztünk a Blastx nevű programmal a génbank (NCBI) adatbázisában. E munkával sikerült mintegy 100 olyan cDNS klónt azonosítani, amelyek szignifikánsan kifejeződtek a fertőzött gazdában, de a fertőzött kontrolban nem. Több olyan gén kifejeződését azonosítottuk, amelyek szekvencia azonosságot mutattak más növények rezisztenciához, illetve stresszhez kapcsolódó génjeivel.

***Cupressus sempervirens* / *Seiridium cardinale* / génkifejeződés / cDNA / gomba kolonizációs profil**

1 INTRODUCTION

Cupressus sempervirens L., (*Cupressaceae*) is native to the Iran, as well as Syria, Turkey, Cyprus and several Greek Islands (Crete, Samos, Rhodes ect.). It was introduced in most countries around Mediterranean and at present its natural geographic distribution is characterised by disjoint and often relic populations growing in Iran, Syria, Jordan, Lebanon, Libya, the Aegean Islands, Crete, Turkey, Cyprus (Zohary 1973). Probably during the Tertiary it occupied a larger areas (Axelrod 1958) that was reduced during millennia mainly by strong human pressure (Boscherini et al. 1994, Vendramin et al. 1995), intensive and unregulated forest utilisation, burning, grazing and cypress canker disease (Kayacik et al. 1979, Sumer 1987, Graniti 1998) leaving only small areas of forest. Since historical times, the cypress has been extensively cultivated far beyond its natural geographic range, in earlier times through its association with religious rites and later for aesthetic reasons. At present it grows also in Italy, France, Spain, Portugal and former Yugoslavia (Ducrey et al. 1999) where it was introduced presumably during the Roman era or even before, since the Phoenicians and Etruscans started to sail along the Mediterranean (Macina 2002). Such spread of cypress is still an ongoing process, not only in Mediterranean countries, but in every similar climatical area too, where the cypress is able to fit to the local environmental conditions (Santini – Di Lonardo 2000, La Porta et al. 2004).

Nowadays, the common cypress has an important role in the characterization of Mediterranean landscape mainly for its aesthetic function. Although there are likely no natural forests of *C. sempervirens* in Italy, this species is adapted very well to the Italian environment and in many sites shows a good natural regeneration to be considered a naturalized species (Ducrey et al. 1999). Cypress groves are present in coastal hills from Liguria to Calabria and in Sicily. In the central part of Italy, especially in Tuscany near Florence, Siena and Pisa, cypress woods are more present and productive. In the north Italy, cypress stands and groves can be mainly found around lakes where climatic conditions are favourable (Xenopoulos 1990, La Porta et al. 2004).

In the last fifty years the cypress has been attacked by a parasitic fungus, *Seiridium cardinale* (canker of cypress), which is seriously threatening the survival of this plant in Italy and in other Mediterranean countries (Panconesi 1990, Graniti 1998). Several cypress improvement programs for resistance were set up with the attempt to cultivate resistant clones throughout wide-reaching territories and areas with highly diverse pedoclimatic conditions. Several resistant clones were actually produced (Panconesi – Raddi 1990, 1991, Danti et al. 2006).

The cypress is a very plastic species: clones growing in completely different habitats take very different shapes in accordance with variations in environmental conditions, ecological

factors and soil characteristics. The strong effect of environment and of environment by genotype interaction on cypress clones has been noted (Santini et al. 1994). Similar conclusions are also being reached in works involving stability in the resistance to cypress canker disease. Clones to use should perhaps be tested locally before spread on a big area, instead of aiming the entire research effort at finding a universal clone adaptable to all environments (Giannini – Raddi 1992).

The low temperatures that the cypress has often to stand in the Italian northern regions act indirectly to increase the strength of penetration of *S. cardinale* spores by means of microlesions created by frost. In this context plants resistant to *S. cardinale* and adapted to cold northern regions guarantee a better protection against pathogen. Cypress clones resistant to canker show also fast recovering of physiological parameters (Muthuchelian et al. 2005a, Muthuchelian et al. 2005b). The presence of highly susceptible hosts and climatic conditions favourable for reproduction of the pathogen facilitated its establishment and spread in the Mediterranean area, where it has caused destructive and recurrent epidemics of canker that have decimated ornamental trees, windbreaks, natural stands and cypress plantations (Graniti 1986, Raddi et al. 1987).

Identification of genes activated or inhibited during the infection process is the basis to better understand the canker resistance. PCR-Select (suppression subtraction hybridization) technique of isolation of genes specific for an infection process, was applied for analysis of host-pathogen interactions in the pathosystem *S. cardinale*/*C. sempervirens*.

The aims of this study were to monitor spatial profiles of the pathogen colonization of susceptible and resistant trees and to identify important genes involved with resistance response to *S. cardinale* infection.

2 MATERIAL AND METHODS

2.1 Plant material and sampling

Two 5 year old clones of *C. sempervirens* growing at the plantation of IASMA Research Centre were used as host material. The two used *C. sempervirens* clones were the resistant patented clone called Bolgheri and a wild clone susceptible to pathogen.

Three resistant ramets and one susceptible ramet were located in greenhouse, thus minimizing the variation in microclimatic conditions. Two resistant ramets and one susceptible clone were inoculated with *S. cardinale*. One resistant ramet was used for control. Prior to inoculation, the fungus was grown on malt extract agar (1% malt extract, 1.5% agar, Difco Laboratories, Detroit, USA) in Petri dishes for 10-15 days at 25°C. Inoculations were made 50 cm above ground level on each stem. At each inoculation point, a plug of bark down to the sapwood surface was excised by using a 5-mm-diameter cork borer. A similarly sized agar plug containing the actively growing fungus was inserted into the hole, and the bark plug was replaced. A rectangular strip (2 x 10 cm) of bark containing phloem and cambium, with the inoculation site in the middle, was removed 7 and 30 days after inoculation. Immediately after excision, the samples were frozen in liquid N_2 and stored at –80°C.

Figure 1. Phases of sampling of the host stem tissue 30 days after inoculations.

2.2 DNA isolation and quantification of fungal colonization

The lesion length was recorded, and the upper half of one resistant and susceptible ramet at 30 dpi were sampled for DNA isolation and Real-Time PCR. Prior to sampling, the rhytidome and periderm were removed. The lesion was divided into sections (length, 1 cm; width, 5 mm; depth, approximately 3 mm), which were processed individually to obtain the fungal colonization profile upper the point inoculation. Fresh tissue was ground in liquid nitrogen with a mortar and a pestle. Genomic DNA was isolated from 50 mg of liquid nitrogen-ground powder by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in agreement with the indications of the producer. The DNA concentration was determined with a Dyna Quant Fluorometer 200 (Hoefer Scientific Instruments, San Fransisco, USA).

The primers wase designed with the Primer Express software, version 1.5, provided with the Applied Biosystems (Foster City, USA) Real-Time quantitative PCR system. A Phytocromo-P gene (GeneBank, AY380891) was used as the target for *C. sempervirens*. The designed forward primer, 5'-CCCGGTTTCTTTTCATGCA-3', and reverse primer, 5'-GGATCAGCACGGCAATCAG-3', amplify the region from base 95 to base 152. A ITS1 gene (GeneBank, AY687314) was used as the target for *S. cardinale*. The designed forward primer, 5'-CGGCGGATTTGTGGTATCC-3', and the reverse primer, 5'-CTGCAGCACCTGACAAAAGC-3', amplify the region from base 458 to base 521.

The Real-Time PCR detection of host and pathogen DNA was performed with SYBR Green PCR Master Mix with an ABI PRISM 7500 Real-Time PCR Instrument (Applied Biosystems).

For optimization of primers concentrations, in order to minimize the interference from competing reactions during multiplex PCR, was performed on samples with both host and fungal material present in known concentrations at a range of dilutions.

The primer concentrations selected were 300 nM of Phitochromo-P primer and 90 nM of ITS1 primer. Each 25 µl PCR was performed in SYBR Green PCR Mastermix (Applied Biosystems, Foster City, USA). As a template, 3 µl of the cDNA solution described above was used for each reaction. Each reaction was repeated twice. PCR cycling parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The data acquisition and analysis were performed with the Sequence Detection System software package (version 1.7; Applied Biosystems, Foster City, USA). The amounts of pathogen and host DNA in the inoculated tissue samples were calculated by using the ΔCt based calculation procedure, with equations derived from the respective standard curve (Hietala et al. 2003).

2.3 RNA extraction and cDNA library

For construction of a forward-subtracted cDNA library, the resistant ramet used as control and a resistant ramet inoculated with *S. cardinale* and incubated for 7 days, were utilised.

Prior to RNA extraction, the rhytidome and the periderm were removed and the tissue subdivided into sections (length, 1 cm; width, 5 mm; depth, about 3 mm) that were processed separately. The sampled 1 cm long sections were frozen immediately in liquid nitrogen and ground with a mortar and a pestle. Approximately 300 mg of the powder was subjected to RNA isolation by following exactly the hot borate extraction method described by Moser et al. (2004). The precipitated RNA was dissolved in a 50 µl volume of RNase-free ddH_2O.

The RNA was purified by using the binding column of SpectrumTM Plant Total RNA Kit (Sigma Aldrich, St. Louis, USA) according to the manufacturer's instructions.

Total RNA was quantified with a VersaFluor fluorometer (Bio-Rad, Hercules, USA) and a RiboGreen RNA quantification kit (Molecular Probes, Eugene, USA) according to the manufacturer's instructions and was DNase-treated by using of Amplification Grade DNaseI (Sigma Aldrich, St. Louis, USA).

cDNA was synthesised from 1 µg of total RNA by using the BD SMART PCR cDNA Synthesis Kit (BD Biosciences, Clontech, East Meadow, USA) according to the manufacturer's instructions. The number of PCR cycles was optimised by LD PCR using 9700 ABI thermal cycler (Applied Biosystems, Foster City, USA) and BD Advantage 2 PCR Kit (BD Biosciences, Clontech, East Meadow, USA) according to the manufacturers' instructions. The optimal cycle number was defined as one cycle fewer than is required to reach the plateau phase. Ten PCR replicates were performed for the two experimental samples (RNA from control ramet and inoculated ramet) by using the obtained optimal cycle number 17. cDNA generated was directly used for construction of a forward-subtracted cDNA library by using Clontech PCR-SelectTM cDNA Subtraction Kit (BDBiosciences, Clontech, East Meadow, USA).

To make the subtracted cDNA library, PCR products from the secondary PCR were directly ligated into the pCR 2.1-TOPO cloning vector (TOPO TA Cloning, Invitrogen, Grand Island, USA) and transformed into One Shot Chemically Competent *E. coli* according to manufacturer's instructions.

For DNA sequencing, each reaction was performed with 4 µl Big dye terminator chemistry (Perkin Elmer, Waltham, USA) in a 20 µl reaction using an ABI Prism 3100 Genetic Analyzer 16 well capillary automated DNA sequencer. Nucleotide sequencing and data analysis were done after deleting the vector sequence. cDNA sequences were compared with GenBank database sequences using BlastX. Sequences for which no match was found were classified as unknown. A subset of interesting clones relevant in host defense and other cellular function was selected for differential screening.

3 RESULTS AND DISCUSSION

The spatial colonization profiles of *S. cardinale* show some difference between the two cypress clones. After 30 days of inoculation, the pathogen was detected until 24 mm high from inoculation point in the susceptible clone. In the resistant clone, it was found only 14 mm high from inoculation point (*Figure 2*). *S. cardinale* colonization levels were very similar in the two clones until the 6th mm from the inoculation point. In this interval, for both clones, there were the maximal amounts of pathogen with a slow decrease. Afterwards, in both clones, there was a rapid decrement in the amount of pathogen between the 6th and 12th mm. In this interval there was a relevant difference in the trend of colonization levels for two clones. At the 8th mm the amount of pathogen's DNA detected in the susceptible clone was about 20 times more present than in the resistant clone. Than, the pathogen was detected until the 14th mm in resistant clone, whereas in the susceptible clone with the regular and constant trend at down levels was found until the 24th mm.

The results of this spatial colonization profile of *S. cardinale* were used to detect the right distance of site where to make genes expression analysis. The sampled section was 6 mm away from the point of inoculation. We assumed that in this area it would be the greatest plant reaction to the pathogen, because we detected a progressive decrement of pathogen DNA.

Moreover, Real Time PCR technique on cypress to make spatial colonization profiles it will be an useful method for the development of early screening able to detect resistant cypress clones.

Expression profile of genes induced by *S. cardinale* in cypress was obtained by sequencing 120 subtractive PCR clones generated from mRNA of bark inoculated with the fungus after subtraction with that of non-inoculated bark. A subset of interesting genes representing important functional categories relevant in host defense and other cellular function from the cDNA library were selected. About 31% of the cDNA sequences had homology to known genes (*Table 1*).

In particular the sequences of chalcone synthase and thioredoxin-dependent peroxidase were obtained from three different PCR clones.

The peroxidases constitute an important group of enzymes associated with phenolic chemistry that are involved in defense-related processes such as lignification, cross-linking of cell wall proteins, auxin catabolism, production of oxygen radicals, as well as direct defense against pathogens (Campa 1991, Mohan et al. 1993, Otter – Polle 1997). Increased accumulation of peroxidases has been reported in several coniferous seedlings infected with pathogenic fungus (Asiegbu et al. 1999, Fossdal et al. 2001, Hietala et al. 2004).

Another interesting enzyme found is the chalcone synthase (CHS) catalyses, the first committed step in the biosynthesis of flavonoids. Flavonoids are implicated in several physiological functions and play a vital role in the interaction between plants and their environment. Stilbenes and flavonoids, together with the enzymes involved in their synthesis (e.g., PAL = phenylalanine ammonia lyase, CHS = chalcone synthase and STS = stilbene synthase; Franceschi et al. 1998, Chiron et al. 2000, Nagy et al. 2000, Viiri et al. 2001), are among the most intensively studied anti-fungal phenolic compounds in coniferous bark. Stilbenes and flavonoids, which are constitutively present as glycosides, represent a primary chemical barrier to invasion and are directly involved in defense against injury and fungal infection (Nicholson - Hammerschmidt 1992, Brignolas et al. 1995, Evensen et al. 2000).

This preliminary results could be useful to identify which genes could change its expression gene levels in responding to *Seridium* infection.

In a further step, a characterization of the most promising candidate genes, particularly chalcone synthase and peroxidase, will be performing by using of Real Time PCR technique on resistant and susceptible cypress clones at different inoculation times.

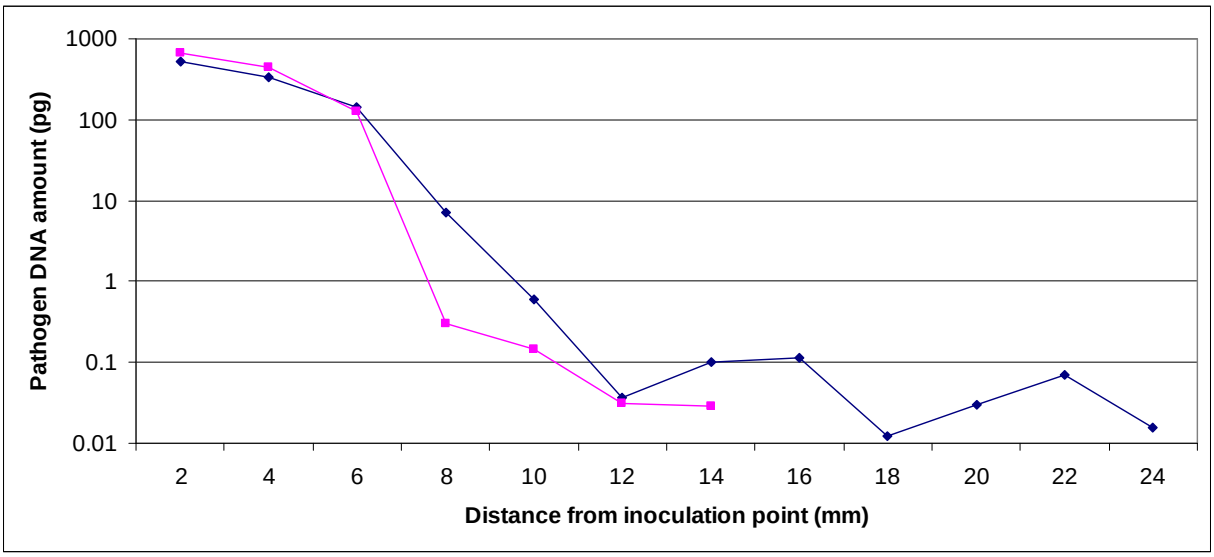

Figure 2. Total DNA yield from the sampled 1 cm-long slices covering the lesions. The lengths of the sampled lesions for resistant (pink) and susceptible (blue) clone.

Table 1. Features of some sequenced clones and results of BLAST search.

CODE	ACCESSION NUMBER	DNA HOMOLOGY	SPECIES	SIZE bp	SCORE	IDENTITIES	E-VALUE
L6	gi|21405653|gb|AY086929.1|	clone 29662 mRNA	*Arabidopsis thaliana*	1084	224 bits (113)	454/565 (80%)	6e-55
L12	gi|71000472|dbj|AB221012.1|	S-adenosyl-L-homocysteine hydrolase	*Beta vulgaris*	424	115 bits (58)	266/323 (82%)	1e-44
E13	gi|45477376|gb|AY559080.1|	LOS2 (los2) mRNA	*Capsella bursa-pastoris*	651	170 bits (86)	260/318 (81%)	5e-39
A15	gi|124358702|dbj|AB264257.1|	CC2188 gene for putative Adomet decarboxylase	*Chamaecyparis obtusa*	614	339 bits (171)	267/299 (89%)	8e-90
H10	gi|90704802|dbj|AB254820.1|	mRNA for putative peptidyl-prolyl cis-trans isomerase	*Cryptomeria japonica*	544	531 bits (268)	429/477 (89%)	9e-148
A17	gi|91075913|gb|DQ395330.2|	alkaline alpha galactosidase (AGA2)	*Cucumis sativus*	1062	81 bits (41)	86/101 (85%)	5e-12
F5	gi|20513302|dbj|AB075535.1|	Chs gene for chalcone syntase Chs	*Juniperus rigida*	405	268 bits (135)	213/239 (89%)	2e-68
F6	gi|20513302|dbj|AB075535.1|	Chs gene for chalcone syntase Chs	*Juniperus rigida*	449	426 bits (215)	323/359 (89%)	3e-116
H20	gi|20513302|dbj|AB075535.1|	Chs gene for chalcone syntase Chs	*Juniperus rigida*	333	280 bits (141)	219/245 (89%)	3e-72
I1	gi|109138844|gb|DQ629331.1|	Qiu 96270 large subunit ribosomal RNA gene	*Juniperus sp.*	432	775 bits (391)	399/402 (99%)	4e-23
E1	gi|924740|gb|U24586.1|	chloroplast 16S rRNA gene	*Juniperus virginiana*	410	670 bits (338)	365/370 (98%)	3e-13
K7	gi|924740|gb|U24586.1|	chloroplast 16S rRNA gene	*Juniperus virginiana*	402	712 bits (359)	373/375 (99%)	6e-18
I17	gi|56554971|gb|AY830127.1|	heat shock protein 70 (HSP70-1) mRNA	*Medicago sativa*	251	127 bits (64)	169/204 (82%)	2e-26
L19	gi|77993048|emb|CR955004.2|	chromosome 5 clone mte1-9e19	*Medicago truncatula*	878	83 bits (42)	93/110 (84%)	1e-12
F21	gi|92898848|gb|AC141323.9|	clone mth2-6a23	*Medicago truncatula*	1042	63 bits (32)	50/56 (89%)	1e-06
M23	gi|77993048|emb|CR955004.2|	chromosome 5 clone mte1-9e19	*Medicago truncatula*	875	83 bits (42)	93/110 (84%)	1e-12
B13	gi|2501849|gb|AF012823.1|	GDP dissociation inhibitor (GDI) mRNA	*Nicotiana tabacum*	546	186 bits (94)	387/479 (80%)	6e-44
M15	gi|51949799|gb|AY695052.1|	adenosine kinase isoform 1S mRNA	*Nicotiana tabacum*	625	167 bits (84)	228/276 (82%)	7e-38
M1	gi|115456202|ref|NM_001058237.1|	chromosome 1	*Oryza sativa*	512	73 bits (37)	58/65 (89%)	6e-10
A11	gi|115474008|ref|NM_001067138.1|	chromosome 7	*Oryza sativa*	733	54 bits (27)	30/31 (96%)	8e-04
E19	gi|115462424|ref|NM_001061347.1|	chromosome 5	*Oryza sativa*	1142	79 bits (40)	79/92 (85%)	2e-11
N21	gi|115474006|ref|NM_001067137.1|	chromosome 7	*Oryza sativa*	813	105 bits (53)	89/101 (88%)	3e-19
A23	gi|52788389|gb|AY705795.1|	clone 1R aldehyde dehydrogenase mRNA	*Pinus halepensis*	886	137 bits (69)	126/144 (87%)	9e-29
B22	gi|4138350|emb|AJ005119.1|	glutamine synhtetase	*Pinus sylvestris*	690	327 bits (165)	352/413 (85%)	3e-86
E7	gi|6752881|gb|AF220200.1|	nascent polypeptide associated complex alpha chain	*Pinus taeda*	756	115 bits (58)	160/194 (82%)	3e-22
D6	gi|6752881|gb|AF220200.1|	nascent polypeptide associated complex alpha chain mRNA	*Pinus taeda*	763	291 bits (147)	390/471 (82%)	2e-75
I11	gi|52851171|emb|AJ843119.1|	mRNA for thioredoxin-dependent peroxidase (tpx1 gene)	*Plantago major*	539	172 bits (87)	198/235 (84%)	9e-40
I15	gi|52851171|emb|AJ843119.1|	mRNA for thioredoxin-dependent peroxidase (tpx1 gene)	*Plantago major*	538	172 bits (87)	198/235 (84%)	9e-40
I7	gi|52851171|emb|AJ843119.1|	mRNA for thioredoxin-dependent peroxidase (tpx1 gene)	*Plantago major*	538	172 bits (87)	198/235 (84%)	9e-40
D11	gi|169704|gb|M64737.1|	ATP:pyruvate phosphotransferase (PK-p-beta) mRNA	*Ricinus communis*	925	127 bits (64)	358/456 (78%)	9e-26
L2	gi|14031062|gb|AY032884.1|	WD-40 repeat protein mRNA	*Solanum lycopersicum*	451	151 bits (76)	214/260 (82%)	3e-33
L3	gi|14031062|gb|AY032884.1|	WD-40 repeat protein mRNA	*Solanum lycopersicum*	445	151 bits (76)	214/260 (82%)	3e-33
G23	gi|410485|emb|Z21792.1|	DAHP synthase 1 precursor	*Solanum lycopersicum*	526	107 bits (54)	261/330 (79%)	5e-20
B1	gi|148538060|dbj|AK246826.1|	ribosomal protein	*Solanum lycopersicum*	923	97 bits (49)	106/125 (84%)	8e-17
L10	gi|13936692|gb|AF295670.1|	plastidic 6-phosphogluconate dehydrogenase (pgdP) mRNA	*Spinacia oleracea*	1188	107 bits (54)	99/114 (86%)	1e-19
D16	gi|147862488|emb|AM484542.2|	contig VV78X020249.4	*Vitis vinifera*	623	77 bits (39)	84/99 (84%)	5e-11

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