EXPRESSION OF PLASMINOGEN AND MICROPLASMINOGEN IN DUCKWEED

FIELD OF THE INVENTION

The present invention relates to recombinant protein production systems. More specifically, the invention is directed to methods and compositions for use in the expression of recombinant plasminogen and recombinant microplasminogen in duckweed.

BACKGROUND OF THE INVENTION

The duckweeds are the sole members of the monocotyledonous family Lemnaceae. The five genera and 38 species are all small, free-floating, fresh-water plants whose geographical range spans the entire globe (Landolt (1986) *Biosystematic Investigation on the Family of Duckweeds: The Family of Lemnaceae—A Monograph Study* Geobatanischen Institut ETH, Stiftung Rubel, Zurich). Although the most morphologically reduced plants known, most duckweed species have all the tissues and organs of much larger plants, including roots, stems, flowers, seeds and fronds. Duckweed species have been studied extensively and a substantial literature exists detailing their ecology, systematics, life-cycle, metabolism, disease and pest susceptibility, their reproductive biology, genetic structure, and cell biology. (Hillman (1961) *Bot. Review* 27: 221; Landolt (1986) *Biosystematic Investigation on the Family of Duckweeds: The Family of Lemnaceae—A Monograph Study* Geobatanischen Institut ETH, Stiftung Rubel, Zurich).

The growth habit of the duckweeds is ideal for microbial culturing methods. The plant rapidly proliferates through vegetative budding of new fronds, in a macroscopic manner analogous to asexual propagation in yeast. This proliferation occurs by vegetative budding from meristematic cells. The meristematic region is small and is found on the ventral surface of the frond. Meristematic cells lie in two

pockets, one on each side of the frond midvein. The small midvein region is also the site from which the root originates and the stem arises that connects each frond to its mother frond. The meristematic pocket is protected by a tissue flap. Fronds bud alternately from these pockets. Doubling times vary by species and are as short as 20-24 hours (Landolt (1957) *Ber. Schweiz. Bot. Ges.* 67: 271; Chang *et al.* (1977) *Bull. Inst. Chem. Acad. Sin.* 24:19; Datko and Mudd (1970) *Plant Physiol.* 65:16; Venkataraman *et al.* (1970) *Z. Pflanzenphysiol.* 62: 316).

Intensive culture of duckweed results in the highest rates of biomass accumulation per unit time (Landolt and Kandeler (1987) *The Family of Lemnaceae—A Monographic Study Vol. 2: Phytochemistry, Physiology, Application, Bibliography*, Veroffentlichungen des Geobotanischen Institutes ETH, Stiftung Rubel, Zurich), with dry weight accumulation ranging from 6-15% of fresh weight (Tillberg *et al.* (1979) *Physiol. Plant.* 46:5; Landolt (1957) *Ber. Schweiz. Bot. Ges.* 67:271; Stomp, unpublished data). Protein content of a number of duckweed species grown under varying conditions has been reported to range from 15-45% dry weight (Chang et al (1977) *Bull. Inst. Chem. Acad. Sin.* 24:19; Chang and Chui (1978) *Z. Pflanzenphysiol.* 89:91; Porath *et al.* (1979) *Aquatic Botany* 7:272; Appenroth *et al.* (1982) *Biochem. Physiol. Pflanz.* 177:251). Using these values, the level of protein production per liter of medium in duckweed is on the same order of magnitude as yeast gene expression systems.

Duckweed plant or duckweed nodule cultures can be efficiently transformed with an expression cassette containing a nucleotide sequence of interest by any one of a number of methods including *Agrobacterium*-mediated gene transfer, ballistic bombardment, or electroporation. Stable duckweed transformants can be isolated by transforming the duckweed cells with both the nucleotide sequence of interest and a gene that confers resistance to a selection agent, followed by culturing the transformed cells in a medium containing the selection agent. See U.S. Patent No. 6,040,498 to Stomp *et al.*

A duckweed gene expression system provides the pivotal technology that would be useful for a number of research and commercial applications. For plant molecular biology research as a whole, a differentiated plant system that can be manipulated with the laboratory convenience of yeast provides a very fast system in

which to analyze the developmental and physiological roles of isolated genes. For commercial production of valuable polypeptides, a duckweed-based system has a number of advantages over existing microbial or cell culture systems. Plants demonstrate post-translational processing that is similar to mammalian cells, overcoming one major problem associated with the microbial cell production of biologically active mammalian polypeptides, and it has been shown by others (Hiatt (1990) *Nature* 334:469) that plant systems have the ability to assemble multi-subunit proteins, an ability often lacking in microbial systems. Scale-up of duckweed biomass to levels necessary for commercial production of recombinant proteins is faster and more cost efficient than similar scale-up of mammalian cells, and unlike other suggested plant production systems, e.g., soybeans and tobacco, duckweed can be grown in fully contained and controlled biomass production vessels, making the system's integration into existing protein production industrial infrastructure far easier.

Accordingly, there remains a need for optimized methods and compositions for expressing proteins of interest in duckweed.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for the production of recombinant plasminogen, microplasminogen, and fragments thereof in a duckweed expression system. The duckweed expression system of the present invention is optimized to produce high levels of plasminogen, microplasminogen, and fragments thereof. The duckweed-produced plasminogen and microplasminogen can be activated to produce a polypeptide having protease activity. Thus, the invention encompasses methods for the expression of plasminogen and microplasminogen in duckweed, duckweed plants that are transformed with expression cassettes for the expression of plasminogen and microplasminogen, and nucleic acids comprising nucleotide sequences encoding plasminogen and microplasminogen, where these nucleotide sequences are modified to enhance their expression in duckweed.

Accordingly, in one embodiment, the present invention provides a method for producing plasminogen in duckweed, wherein said method comprises the steps of culturing a duckweed plant or duckweed plant cell, where the duckweed plant or

duckweed plant cell is stably transformed with a nucleic acid molecule comprising a nucleotide sequence encoding plasminogen; and collecting the plasminogen from said duckweed plant or duckweed plant cell. In some embodiments, the nucleotide sequence encoding plasminogen is operably linked to a nucleotide sequence encoding a signal peptide.

In another embodiment, the present invention provides a method for producing microplasminogen in duckweed, where the method comprises the steps of culturing within a duckweed culture medium a duckweed plant culture or a duckweed nodule culture, where the duckweed plant culture or the duckweed nodule culture is stably transformed with a nucleic acid molecule comprising a nucleotide sequence encoding microplasminogen and an operably linked coding sequence for a signal peptide that directs secretion of the microplasminogen into the culture medium; and collecting the microplasminogen from the duckweed culture medium.

The invention also encompasses duckweed plants, duckweed nodules, and duckweed plant cells transformed with expression cassettes capable of expressing plasminogen or microplasminogen in duckweed. Also provided are nucleic acid molecules comprising a nucleotide sequence encoding plasminogen or microplasminogen, where the nucleotide sequences comprise duckweed-optimized codons.

These and other aspects of the present invention are disclosed in more detail in the description of the invention given below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the level of plasminogen in tissue homogenates measured by ELISA in 56 duckweed lines that were transformed with the plasminogen expression construct BAP01. The level of plasminogen is expressed as a percentage of the total soluble protein in the homogenates. See Example 1 in the Experimental section for additional details.

Figure 2 shows the activity of plasmin produced by activating duckweed-produced plasminogen with tPA. See Example 1 in the Experimental section for additional details.

Figure 3 shows the quantitation of plasminogen by ELISA compared to plasmin activity after streptokinase activation. For the lines tested, quantitation by ELISA and streptokinase activity assay gave comparable values, indicating that *Lemna*-produced plasminogen has a similar specific activity to the control protein. See Example 1 in the Experimental section for additional details.

Figure 4 shows the stability of human plasminogen spiked into Lemna tissue extract. See Example 1 in the Experimental section for additional details.

Figure 5 shows the stability of Lemna plasminogen in tissue extract after freeze/thaw as measured by an activity assay. See Example 1 in the Experimental section for additional details.

Figure 6 shows the formation of plasmin after urokinase or tPA activation of *Lemna*-produced plasminogen. "BAP" designates the *Lemna*-produced plasminogen. The left panel shows activation with tissue plasminogen activator (tPA) and the right panel shows activation with urokinase (uK). See Example 1 in the Experimental section for additional details.

Figure 7 shows the concentration of microplasminogen as measured by ELISA in media from 79 duckweed lines that were transformed with the microplasminogen expression construct BAMP01. See Example 2 in the Experimental section for additional details.

Figure 8 shows a zymogram analysis of *Lemna*-produced microplasminogen after activation by tPA. The figure shows the presence of an active proteolytic band that is not present in concentrated control media. See Example 2 in the Experimental section for additional details.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is drawn to methods and compositions for the production of recombinant plasminogen, microplasminogen, and fragments thereof in a duckweed expression system. It is the novel finding of the present invention that a duckweed expression system may be used to produce high levels of plasminogen and microplasminogen. The duckweed-produced plasminogen and microplasminogen can be activated to produce a polypeptide having protease activity.

Plasminogen is the inactive precursor form of plasmin, the principal fibrinolytic enzyme in mammals. Plasmin also plays an important role in cell migration, tissue remodeling, and bacterial invasion. Plasmin is a serine protease that preferentially cleaves Lys-|-Xaa and Arg-|-Xaa bonds with higher selectivity than trypsin. Plasminogen activators such as tissue plasminogen activator (tPA) or urokinase cleave human plasminogen molecule at the Arg_{560}-Val_{561} bond to produce active plasmin. The two resulting chains of plasmin are held together by two interchain disulphide bridges. The light chain (25 kDa) carries the catalytic center (which comprises the catalytic triad) and shares sequence similarity with trypsin and other serine proteases. The heavy chain (60 kDa) consists of five highly similar triple-loop structures called kringles. Some of the kringles contain lysine binding sites that mediates the plasminogen/plasmin interaction with fibrin. Plasmin belongs to peptidase family S1.

Microplasminogen consists of the proenzyme domain of plasminogen with a stretch of connecting peptide and a few residues of kringle 5 attached at its N-terminal end. It is produced by the action of plasmin on plasminogen. *See*, for example, Shi *et al.* (1980) *J. Biol. Chem.* 263:17071-5. Like plasminogen, microplasminogen is activated by tPA and urokinase to form a proteolytically active molecule. Human microplasmin has a molecular weight of approximately 29 kDa and has a lower affinity for fibrin when compared with plasmin.

Plasmin and microplasmin are proposed for use in thrombolytic therapy in a number of applications including the treatment of myocardial infarction, occlusive stroke, deep venous thrombosis, and peripheral arterial diseases. See, for example, U.S. Patent No. 5,407,673, U.S. Patent No. 6,355,243, U.S. Patent Application No. 20030175264, Lapchak *et al.* (2002) *Stroke* 33:2279-2284, and Nagai *et al.* (2003) *J. Thromb. Haemost.* 1:307-13, each of which is herein incorporated by reference in its entirety. One goal of using plasmin and microplasmin in such therapy is to avoid the side effects of therapy using plasminogen activators such as tPA, urokinase, and streptokinase. Such side effects include gastrointestinal and intercranial hemorrhage. However, the use of plasmin and microplasmin as therapeutic agents has been limited in part by the difficulty of producing large quantities of stable plasminogen and microplasminogen precursor proteins.

Although expression of large amounts of plasminogen in a recombinant system is a convenient way to obtain plasminogen for use in thrombolytic therapy, there have been great difficulties in expression of intact human plasminogen in expression systems due to the nearly ubiquitous presence of intracellular plasminogen activators in mammalian cell types. The presence of these activators results in the degradation of the produced plasminogen. See, for example, Busy *et al.* (1988) *Fibrinolysis* 2:64.

The present invention solves this problem by providing an expression system capable of expressing high levels of stable plasminogen and microplasminogen. Thus, in one embodiment, the present invention provides a method for producing plasminogen in duckweed, wherein said method comprises the steps of culturing a duckweed plant or duckweed plant cell, where the duckweed plant or duckweed plant cell is stably transformed with a nucleic acid molecule comprising a nucleotide sequence encoding plasminogen; and collecting the plasminogen from said duckweed plant or duckweed plant cell. The nucleotide sequence encoding plasminogen may be operably linked to a nucleotide sequence encoding a signal peptide.

The methods of the invention may be used to express high levels of plasminogen in duckweed. Thus, in some embodiments of the method, at least about 1%, at least about 2%, at least about 3%, at least about 4%, at least about 5%, at least about 6%, at least about 7%, or at least about 8% of the soluble protein in the duckweed plant or duckweed plant cell is plasminogen.

The present invention also provides an improvement in a method of producing stable plasminogen in duckweed, wherein the improvement comprises producing the plasminogen in duckweed. The *Lemna*-produced plasminogen is stable and loses less than 10% of its activity when stored overnight in a *Lemna* tissue extract. The *Lemna*-produced plasminogen also undergoes less than 10% degradation in *Lemna* tissue extracts following a freeze-thaw cycle.

In another embodiment, the present invention provides a method for producing microplasminogen in duckweed, where the method comprises the steps of culturing within a duckweed culture medium a duckweed plant culture or a duckweed nodule culture, where the duckweed plant culture or the duckweed nodule culture is stably transformed with a nucleic acid molecule comprising a nucleotide sequence encoding

microplasminogen and an operably linked coding sequence for a signal peptide that directs secretion of the microplasminogen into the culture medium; and collecting the microplasminogen from the duckweed culture medium.

The methods of the invention may be used to express high levels of microplasminogen in duckweed. Thus, in some embodiments of the method, the, duckweed culture medium contains at least about 1 mg/L, at least about 2 mg/L, at least about 5 mg/L microplasminogen, at least about 10 mg/L microplasminogen, at least about 15 mg/L microplasminogen, or at least about 20 mg/L microplasminogen as determined by quantitative Western blotting.

The present invention provides a method for producing a plasminogen fragment in duckweed, wherein said method comprises the steps of culturing a duckweed plant or duckweed plant cell, where the duckweed plant or duckweed plant cell is stably transformed with a nucleic acid molecule comprising a nucleotide sequence encoding the plasminogen fragment; and collecting the plasminogen fragment from the duckweed plant, the duckweed plant cell, or the duckweed culture medium. The nucleotide sequence encoding plasminogen may be operably linked to a nucleotide sequence encoding a signal peptide.

In some embodiments of the methods of the present invention, the nucleic acid molecule comprising the nucleotide sequence encoding plasminogen, microplasminogen, or fragment thereof is modified to enhance its expression in duckweed. Examples of such modifications include the use of duckweed-preferred codons in the coding sequence for the plasminogen microplasminogen, the use of an operably linked nucleotide sequence comprising a plant intron that is inserted upstream of the coding sequence; and the use of a leader sequence that increases the translation of the nucleotide sequence encoding plasminogen or microplasminogen. In some embodiments, two or more of these modifications are used in combination. Where the nucleotide sequence encoding plasminogen is operably linked to a nucleotide sequence encoding a signal peptide, the nucleotide sequence encoding the signal peptide may also comprise duckweed-preferred codons.

The invention also encompasses duckweed plants, duckweed nodules, and duckweed plant cells transformed with expression cassettes capable of expressing plasminogen or microplasminogen in duckweed. Also provided are nucleic acid

molecules comprising a nucleotide sequence encoding plasminogen or microplasminogen, where the nucleotide sequences comprise duckweed-optimized codons.

Definitions:

"Polypeptide" refers to any monomeric or multimeric protein or peptide.

"Biologically active polypeptide" refers to a polypeptide that has the capability of performing one or more biological functions or a set of activities normally attributed to the polypeptide in a biological context. Within the context of a protease precursor such as plasminogen or microplasminogen, biological activity encompasses the capability of the polypeptide to be activated to produce a proteolytically active molecule. Proteolytic activity of plasminogen or microplasminogen following activation may be measured by any assay known in the art, including the assay based on the use of a chromogenic substrate as described elsewhere herein.

The terms "expression" or "production" refer to the biosynthesis of a gene product, including the transcription, translation, and assembly of said gene product.

The term "duckweed" refers to members of the family Lemnaceae. This family currently is divided into five genera and 38 species of duckweed as follows: genus *Lemna* (*L. aequinoctialis*, *L. disperma*, *L. ecuadoriensis*, *L. gibba*, *L. japonica*, *L. minor*, *L. miniscula*, *L. obscura*, *L. perpusilla*, *L. tenera*, *L. trisulca*, *L. turionifera*, *L. valdiviana*); genus *Spirodela* (*S. intermedia*, *S. polyrrhiza*, *S. punctata*); genus *Wolffia* (*Wa. angusta*, *Wa. arrhiza*, *Wa. australina*, *Wa. borealis*, *Wa. brasiliensis*, *Wa. columbiana*, *Wa. elongata*, *Wa. globosa*, *Wa. microscopica*, *Wa. neglecta*);genus *Wolfiella* (*Wl. caudata*, *Wl. denticulata*, *Wl. gladiata*, *Wl. hyalina*, *Wl. lingulata*, *Wl. repunda*, *Wl. rotunda*, and *Wl. neotropica*) and genus *Landoltia (L. punctata)*. Any other genera or species of Lemnaceae, if they exist, are also aspects of the present invention. *Lemna* species can be classified using the taxonomic scheme described by Landolt (1986) *Biosystematic Investigation on the Family of Duckweeds: The family of Lemnaceae—A Monograph Study* Geobatanischen Institut ETH, Stiftung Rubel, Zurich.

The term "duckweed nodule culture" as used herein refers to a culture comprising duckweed cells wherein at least about 50%, 55%, 60%, 65%, 70%, 75%,

80%, 85%, 90%, 95%, or 100% of the cells are differentiated cells. A "differentiated cell," as used herein, is a cell with at least one phenotypic characteristic (e.g., a distinctive cell morphology or the expression of a marker nucleic acid or protein) that distinguishes it from undifferentiated cells or from cells found in other tissue types. The differentiated cells of the duckweed nodule culture described herein form a tiled smooth surface of interconnected cells fused at their adjacent cell walls, with nodules that have begun to organize into frond primordium scattered throughout the tissue. The surface of the tissue of the nodule culture has epidermal cells connect to each other via plasmadesmata.

"Duckweed-preferred codons" as used herein refers to codons that have a frequency of codon usage in duckweed of greater than 17%.

"*Lemna*-preferred codons" as used herein refers to codons that have a frequency of codon usage in the genus *Lemna* of greater than 17%.

"*Lemna gibba*-preferred codons" as used herein refers to codons that have a frequency of codon usage in *Lemna gibba* of greater than 17%.

"Translation initiation codon" refers to the codon that initiates the translation of the mRNA transcribed from the nucleotide sequence of interest.

"Translation initiation context nucleotide sequence" as used herein refers to the identity of the three nucleotides directly 5' of the translation initiation codon.

"Secretion" as used herein refers to translocation of a polypeptide across the plasma membrane of a host plant cell. In some embodiments of the present invention, the polypeptide is retained within the apoplast, the region between the plasma membrane and the cell wall. In other embodiments, the polypeptide is translocated across cell wall of the plant host cell.

"Operably linked" as used herein in reference to nucleotide sequences refers to multiple nucleotide sequences that are placed in a functional relationship with each other. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in reading frame.

A. Expression Cassettes

According to the present invention, stably transformed duckweed is obtained by transformation with an expression cassette comprising a nucleotide sequence

encoding plasminogen or a nucleotide sequence encoding microplasminogen. The expression cassette comprises a transcriptional initiation region linked to the nucleic acid or gene of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the nucleotide sequence encoding the protein of interest to be under the transcriptional regulation of the regulatory regions. The expression cassette may encode a single gene of interest. In particular embodiments of the invention, the nucleic acid to be transferred contains two or more expression cassettes, each of which encodes at least one gene of interest.

The transcriptional initiation region, (e.g., a promoter) may be native or homologous or foreign or heterologous to the host, or could be the natural sequence or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced. As used herein a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

Any suitable promoter known in the art can be employed according to the present invention (including bacterial, yeast, fungal, insect, mammalian, and plant promoters). For example, plant promoters, including duckweed promoters, may be used. Exemplary promoters include, but are not limited to, the Cauliflower Mosaic Virus 35S promoter, the opine synthetase promoters (e.g., nos, mas, ocs, etc.), the ubiquitin promoter, the actin promoter, the ribulose bisphosphate (RubP) carboxylase small subunit promoter, and the alcohol dehydrogenase promoter. The duckweed RubP carboxylase small subunit promoter is known in the art (Silverthorne *et al.* (1990) *Plant Mol. Biol.* 15:49). Other promoters from viruses that infect plants, preferably duckweed, are also suitable including, but not limited to, promoters isolated from Dasheen mosaic virus, Chlorella virus (e.g., the Chlorella virus adenine methyltransferase promoter; Mitra *et al.* (1994) *Plant Mol. Biol.* 26:85), tomato spotted wilt virus, tobacco rattle virus, tobacco necrosis virus, tobacco ring spot virus, tomato ring spot virus, cucumber mosaic virus, peanut stump virus, alfalfa mosaic virus, sugarcane baciliform badnavirus and the like.

Finally, promoters can be chosen to give a desired level of regulation. For example, in some instances, it may be advantageous to use a promoter that confers

constitutive expression (e.g, the mannopine synthase promoter from *Agrobacterium tumefaciens*). Alternatively, in other situations, it may be advantageous to use promoters that are activated in response to specific environmental stimuli (e.g., heat shock gene promoters, drought-inducible gene promoters, pathogen-inducible gene promoters, wound-inducible gene promoters, and light/dark-inducible gene promoters) or plant growth regulators (e.g., promoters from genes induced by abscissic acid, auxins, cytokinins, and gibberellic acid) or other compounds such as ethanol or ethylene. As a further alternative, promoters can be chosen that give tissue-specific expression (e.g., root, leaf, and floral-specific promoters).

The overall strength of a given promoter can be influenced by the combination and spatial organization of cis-acting nucleotide sequences such as upstream activating sequences. For example, activating nucleotide sequences derived from the *Agrobacterium tumefaciens* octopine synthase gene can enhance transcription from the *Agrobacterium tumefaciens* mannopine synthase promoter (see U.S. Patent 5,955,646 to Gelvin *et al.*). In the present invention, the expression cassette can contain activating nucleotide sequences inserted upstream of the promoter sequence to enhance the expression of the nucleotide sequence of interest. In one embodiment, the expression cassette includes three upstream activating sequences derived from the *Agrobacterium tumefaciens* octopine synthase gene operably linked to a promoter derived from an *Agrobacterium tumefaciens* mannopine synthase gene (see U.S Patent 5,955,646, herein incorporated by reference).

The transcriptional cassette includes in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a nucleotide sequence of interest, and a transcriptional and translational termination region functional in plants. Any suitable termination sequence known in the art may be used in accordance with the present invention. The termination region may be native with the transcriptional initiation region, may be native with the nucleotide sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid *of A. tumefaciens*, such as the octopine synthetase and nopaline synthetase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141; Proudfoot (1991) *Cell* 64:671; Sanfacon *et al.* (1991) *Genes Dev.* 5:141; Mogen *et al.* (1990) *Plant Cell* 2:1261; Munroe *et al.* (1990) *Gene* 91:151; Ballas *et al.* (1989)

Nucleic Acids Res. 17:7891; and Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627. Additional exemplary termination sequences are the pea RubP carboxylase small subunit termination sequence and the Cauliflower Mosaic Virus 35S termination sequence and the ubiquitin terminator from many plant species. Other suitable termination sequences will be apparent to those skilled in the art.

The expression cassettes may contain more than one gene or nucleic acid sequence to be transferred and expressed in the transformed plant. Thus, each nucleic acid sequence will be operably linked to 5' and 3' regulatory sequences. Alternatively, multiple expression cassettes may be provided.

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells or tissues. Selectable marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO), neomycin phosphotransferase III and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds. Herbicide resistance genes generally code for a modified target protein insensitive to the herbicide or for an enzyme that degrades or detoxifies the herbicide in the plant before it can act. See DeBlock *et al.* (1987) *EMBO J.* 6:2513; DeBlock *et al.*(1989) *Plant Physiol.* 91:691; Fromm *et al.* (1990) *BioTechnology* 8:833; Gordon-Kamm *et al.* (1990) *Plant Cell* 2:603; and Frisch *et al.* (1995) *Plant Mol. Biol.* 27:405-9. For example, resistance to glyphosphate or sulfonylurea herbicides has been obtained using genes coding for the mutant target enzymes, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and acetolactate synthase (ALS). Resistance to glufosinate ammonium, boromoxynil, and 2,4-dichlorophenoxyacetate (2,4-D) have been obtained by using bacterial genes encoding phosphinothricin acetyltransferase, a nitrilase, or a 2,4-dichlorophenoxyacetate monooxygenase, which detoxify the respective herbicides.

For purposes of the present invention, selectable marker genes include, but are not limited to, genes encoding neomycin phosphotransferase II (Fraley *et al.* (1986) *CRC Critical Reviews in Plant Science* 4:1), neomycin phosphotransferase III (Frisch *et al.* (1995) *Plant Mol. Biol.* 27:405-9), cyanamide hydratase (Maier-Greiner *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:4250); aspartate kinase; dihydrodipicolinate synthase (Perl *et al.* (1993) *BioTechnology* 11:715); bar gene (Toki *et al.* (1992) *Plant*

Physiol. 100:1503; Meagher *et al.* (1996) *Crop Sci.* 36:1367); tryptophan decarboxylase (Goddijn *et al.* (1993) *Plant Mol. Biol.* 22:907); neomycin phosphotransferase (NEO; Southern *et al.* (1982) *J. Mol. Appl. Gen.* 1:327); hygromycin phosphotransferase (HPT or HYG; Shimizu *et al.* (1986) *Mol. Cell. Biol.* 6:1074); dihydrofolate reductase (DHFR; Kwok *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:4552); phosphinothricin acetyltransferase (DeBlock *et al.* (1987) *EMBO J.* 6:2513); 2,2-dichloropropionic acid dehalogenase (Buchanan-Wollatron *et al.* (1989) *J. Cell. Biochem.* 13D:330); acetohydroxyacid synthase (U.S. Pat. No. 4,761,373 to Anderson *et al.*; Haughn *et al.* (1988) *Mol. Gen. Genet.* 221:266); 5-enolpyruvyl-shikimate-phosphate synthase (aroA; Comai *et al.* (1985) *Nature* 317:741); haloarylnitrilase (WO 87/04181 to Stalker *et al.*); acetyl-coenzyme A carboxylase (Parker *et al.* (1990) *Plant Physiol.* 92:1220); dihydropteroate synthase (sulI; Guerineau *et al.* (1990) *Plant Mol. Biol.* 15:127); and 32 kDa photosystem II polypeptide (psbA; Hirschberg *et al.* (1983) *Science* 222:1346 (1983).

Also included are genes encoding resistance to: gentamycin (*e.g.*, aacC1, Wohlleben *et al.* (1989) *Mol. Gen. Genet.* 217:202-208); chloramphenicol (Herrera-Estrella *et al.* (1983) *EMBO J.* 2:987); methotrexate (Herrera-Estrella *et al.* (1983) *Nature* 303:209; Meijer *et al.* (1991) *Plant Mol. Biol.* 16:807); hygromycin (Waldron *et al.* (1985) *Plant Mol. Biol.* 5:103; Zhijian *et al.* (1995) *Plant Science* 108:219; Meijer *et al.* (1991) *Plant Mol. Bio.* 16:807); streptomycin (Jones *et al.* (1987) *Mol. Gen. Genet.* 210:86); spectinomycin (Bretagne-Sagnard *et al.* (1996) *Transgenic Res.* 5:131); bleomycin (Hille *et al.* (1986) *Plant Mol. Biol.* 7:171); sulfonamide (Guerineau *et al.* (1990) *Plant Mol. Bio.* 15:127); bromoxynil (Stalker *et al.* (1988) *Science* 242:419); 2,4-D (Streber *et al.* (1989) *BioTechnology* 7:811); phosphinothricin (DeBlock *et al.* (1987) EMBO J. 6:2513); spectinomycin (Bretagne-Sagnard and Chupeau, *Transgenic Research* 5:131).

The bar gene confers herbicide resistance to glufosinate-type herbicides, such as phosphinothricin (PPT) or bialaphos, and the like. As noted above, other selectable markers that could be used in the vector constructs include, but are not limited to, the pat gene, also for bialaphos and phosphinothricin resistance, the ALS gene for imidazolinone resistance, the HPH or HYG gene for hygromycin resistance, the EPSP synthase gene for glyphosate resistance, the Hm1 gene for resistance to the Hc-toxin,

and other selective agents used routinely and known to one of ordinary skill in the art. See Yarranton (1992) *Curr. Opin. Biotech.* 3:506; Chistopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314; Yao *et al.* (1992) *Cell* 71:63; Reznikoff (1992) *Mol. Microbiol.* 6:2419; Barkley *et al.* (1980) *The Operon* 177-220; Hu *et al.* (1987) *Cell* 48:555; Brown *et al.* (1987) *Cell* 49:603; Figge *et al.* (1988) *Cell* 52:713; Deuschle *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5400; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549; Deuschle *et al.* (1990) *Science* 248:480; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072; Wyborski *et al.* (1991) *Nuc. Acids Res.* 19:4647; Hillenand-Wissman (1989) *Topics in Mol. And Struc. Biol.* 10:143; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591; Kleinschnidt *et al.* (1988) *Biochemistry* 27:1094; Gatz *et al.* (1992) *Plant J.* 2:397; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology* 78; and Gill *et al.* (1988) *Nature* 334:721. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes are not meant to be limiting. Any lethal or non-lethal selectable marker gene can be used in the present invention.

B. Plasminogen and Microplasminogen

The present invention is directed to methods and compositions for the expression of plasminogen, microplasminogen, and fragments thereof in duckweed. The plasminogen to be expressed in duckweed may be from any mammalian source. In some embodiments, the plasminogen is human or porcine. In a particular embodiment, the plasminogen has the amino acid sequence of the human plasminogen shown in SEQ ID NO:4. In other embodiments, the plasminogen is a biologically active variant of the amino acid sequence shown in SEQ ID NO:4.

Similarly, the microplasminogen to be expressed in duckweed may be from any mammalian source. In some embodiments, the microplasminogen is human or porcine. In a particular embodiment, the microplasminogen has the amino acid sequence of the human microplasminogen shown in SEQ ID NO:6. In other

embodiments, the plasminogen is a biologically active variant of the amino acid sequence shown in SEQ ID NO6.

Fragments of plasminogen or microplasminogen may be produced according to the present invention. Such fragments may comprise at least 50, at least 60, at least 70, at least 80, at least 90, at least 95, at least 101, at least 150, at least 200, at least 250, at least 300, at least 350, at least 377, at least 400, at least 450, at least 500, at least 550, at least 600, at least 650, at least 700, or at least 750 contiguous amino acids of a plasminogen protein. Examples of fragments that may be produced according to the invention include miniplasminogen and angiostatin. Non-limiting examples of plasminogen or microplasminogen fragments are given in O'Reilly *et al.* (1994) *Cell* 79:315-28; Sim *et al.* (1997) *Cancer Res.* 57:1329-34; U.S. Patent 5,972,896, and U.S. Patent Publications 20020164717, 20020037847, and 20010016644; each of which is herein incorporated by reference in its entirety. In some embodiments, the fragments retain the enzymatic activity, for example the protease activity, of plasminogen or microplasminogen.

A "biologically active variant" of plasminogen or microplasminogen is a polypeptide derived from these polypeptides by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the protein; or substitution of one or more amino acids at one or more sites in the protein. Biologically active variant plasminogen and microplasminogen polypeptides encompassed by the present invention are biologically active, that is they are capable of being activated to produce a protein having the protease activity of the plasmin family of proteases (Enzyme Class 3.4.21.7). Such biologically active variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a plasminogen or microplasminogen according to the invention will have at least about 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, such as at least about 98%, 99% or more sequence identity to the amino acid sequence shown in SEQ ID NO:4 or SEQ ID NO:6. Thus, a biologically active variant of plasminogen or microplasminogen of the invention may differ from the amino acid sequences shown in SEQ ID NO: 4 and SEQ ID NO:6 by as few as 1-15 amino acid

residues, as few as 1-10 amino acid residues, such as 6-10 amino acid residues, as few as 5 amino acid residues, or as few as 4, 3, 2, or even 1 amino acid residue. Examples of biologically active variants of plasminogen are known in the art and are described, for example, in U.S. Patent No. 5,190,756. In order to retain biological activity, any substitutions will preferably be conservative in nature, and truncations and substitutions will generally made in residues that are not required for protease activity. The residues and domains underlying the activity of plasmin/plasminogen and microplasmin/microplasminogen are known in the art and have been described, for example, in Kolev *et al.* (1997) *J. Biol. Chem.* 272:13666-675; de los Santos *et al.* (1997) *Ciba Found. Symp.* 212:66-76, Peisach *et al.* (1999) *Biochemistry* 38:11180-11188, and Turner *et al.* (2002) *J. Biol. Chem.* 277:33-68-74); each of which is herein incorporated by reference in its entirety.

The comparison of sequences and determination of percent identity and percent similarity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (1970) *J. Mol. Biol.* *48*:444-453 algorithm, which is incorporated into the GAP program in the GCG software package (available at www.accelrys.com), using either a BLOSSUM62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package, using a BLOSUM62 scoring matrix (see Henikoff *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that should be used if the practitioner is uncertain about what parameters should be applied to determine if a molecule is within a sequence identity limitation of the invention) is using a BLOSUM62 scoring matrix with a gap weight of 60 and a length weight of 3).

The percent identity between two amino acid or nucleotide sequences can also be determined using the algorithm of E. Meyers and W. Miller (1989) *CABIOS 4*:11-17 which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

C. Modification of Nucleotide Sequences for Enhanced Expression in Duckweed

In some embodiments, the present invention provides for the modification of the expressed nucleotide sequence to enhance its expression in duckweed. One such modification is the synthesis of the nucleotide sequence encoding plasminogen or microplasminogen using duckweed-preferred codons. Methods are available in the art for synthesizing nucleotide sequences with plant-preferred codons. See, for example, U.S. Patent Nos. 5,380,831 and 5,436,391; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 15:3324; Iannacome *et al.* (1997) *Plant Mol. Biol.* 34:485; and Murray *et al.*, (1989) *Nucleic Acids. Res.* 17:477, herein incorporated by reference. The preferred codons may be determined from the codons of highest frequency in the proteins expressed in duckweed. Thus, the frequency of usage of particular a codon in duckweed may be determined by analyzing codon usage in a group of duckweed coding sequences. A number of duckweed coding sequences are known to those of skill in the art; see for example, the sequences contained in the GenBank® database which may be accessed through the website for the National Center for Biotechnology Information, a division of the National Library of Medicine, which is located in Bethesda, Maryland. Tables showing the frequency of codon usage based on the sequences contained in the most recent GenBank® release may be found on the website for the Kazusa DNA Research Institute in Chiba Japan; see www.kazusa.or.jp/codon/. This database is described in Nakamura *et al.* (2000) *Nucl. Acids Res.* 28: 292.

It is recognized that genes that have been optimized for expression in duckweed and other monocots can be used in the methods of the invention. See, e.g., EP 0 359 472, EP 0 385 962, WO 91/16432; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:3324; Iannacome *et al.* (1997) *Plant Mol. Biol.* 34:485; and Murray *et al.* (1989) *Nuc. Acids Res.* 17:477, and the like, herein incorporated by reference. It is further recognized that all or any part of the gene sequence may be optimized or synthetic. In other words, fully optimized or partially optimized sequences may also be used. For example, 40 %, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the codons may be duckweed-preferred codons. For example, in some embodiments, the

nucleotide sequence encoding plasminogen or microplasminogen comprises between 50-100% duckweed-preferred codons or between 70-100% duckweed preferred codons. In one embodiment, between 90 and 96 % of the codons are duckweed-preferred codons. The coding sequence of the nucleotide sequence of interest may comprise codons used with a frequency of at least 17% in duckweed. Codon usage in *Lemna gibba* (Table 1) and *Lemna minor* (Table 2) is shown below. In some embodiments, Table 1 or Table 2 is used to select duckweed preferred codons. In particular embodiments, the duckweed codon optimized sequence encoding plasminogen is the sequence shown in SEQ ID NO:3, and the duckweed codon optimized sequence encoding microplasminogen is the nucleotide sequence shown in SEQ ID NO:5.

Table 1: *Lemna gibba* codon usage from GenBank® Release 139*

Amino Acid	Codon	Number	/1000	Fraction
Gly	GGG	57.00	28.89	0.35
Gly	GGA	8.00	4.05	0.05
Gly	GGT	3.00	1.52	0.02
Gly	GGC	93.00	47.14	0.58
Glu	GAG	123.00	62.34	0.95
Glu	GAA	6.00	3.04	0.05
Asp	GAT	6.00	3.04	0.08
Asp	GAC	72.00	36.49	0.92
Val	GTG	62.00	31.42	0.47
Val	GTA	0.00	0.00	0.00
Val	GTT	18.00	9.12	0.14
Val	GTC	51.00	25.85	0.39
Ala	GCG	44.00	22.30	0.21
Ala	GCA	14.00	7.10	0.07
Ala	GCT	14.00	7.10	0.07
Ala	GCC	139.00	70.45	0.66
Arg	AGG	16.00	8.11	0.15
Arg	AGA	11.00	5.58	0.10
Ser	AGT	1.00	0.51	0.01
Ser	AGC	44.00	22.30	0.31
Lys	AAG	116.00	58.79	1.00
Lys	AAA	0.00	0.00	0.00
Asn	AAT	2.00	1.01	0.03
Asn	AAC	70.00	35.48	0.97
Met	ATG	67.00	33.96	1.00
Ile	ATA	4.00	2.03	0.06

Ile	ATT	0.00	0.00	0.00
Ile	ATC	63.00	31.93	0.94
Thr	ACG	19.00	9.63	0.25
Thr	ACA	1.00	0.51	0.01
Thr	ACT	6.00	3.04	0.08
Thr	ACC	50.00	25.34	0.66
Trp	TGG	45.00	22.81	1.00
End	TGA	4.00	2.03	0.36
Cys	TGT	0.00	0.00	0.00
Cys	TGC	34.00	17.23	1.00
End	TAG	0.00	0.00	0.00
End	TAA	7.00	3.55	0.64
Tyr	TAT	4.00	2.03	0.05
Tyr	TAC	76.00	38.52	0.95
Leu	TTG	5.00	2.53	0.04
Leu	TTA	0.00	0.00	0.00
Phe	TTT	4.00	2.03	0.04
Phe	TTC	92.00	46.63	0.96
Ser	TCG	34.00	17.23	0.24
Ser	TCA	2.00	1.01	0.01
Ser	TCT	1.00	0.51	0.01
Ser	TCC	59.00	29.90	0.42
Arg	CGG	23.00	11.66	0.22
Arg	CGA	3.00	1.52	0.03
Arg	CGT	2.00	1.01	0.02
Arg	CGC	50.00	25.34	0.48
Gln	CAG	59.00	29.90	0.86
Gln	CAA	10.00	5.07	0.14
His	CAT	5.00	2.53	0.26
His	CAC	14.00	7.10	0.74
Leu	CTG	43.00	21.79	0.35
Leu	CTA	2.00	1.01	0.02
Leu	CTT	1.00	0.51	0.01
Leu	CTC	71.00	35.99	0.58
Pro	CCG	44.00	22.30	0.31
Pro	CCA	6.00	3.04	0.04
Pro	CCT	13.00	6.59	0.09
Pro	CCC	80.00	40.55	0.56

Table 2: *Lemna minor* codon usage from GenBank® Release 139*

AmAcid	Codon	Number	/1000	Fraction
Gly	GGG	8.00	17.39	0.22
Gly	GGA	11.00	23.91	0.31
Gly	GGT	1.00	2.17	0.03
Gly	GGC	16.00	34.78	0.44
Glu	GAG	25.00	54.35	0.78
Glu	GAA	7.00	15.22	0.22
Asp	GAT	8.00	17.39	0.33
Asp	GAC	16.00	34.78	0.67
Val	GTG	21.00	45.65	0.53
Val	GTA	3.00	6.52	0.07
Val	GTT	6.00	13.04	0.15
Val	GTC	10.00	21.74	0.25
Ala	GCG	13.00	28.26	0.32
Ala	GCA	8.00	17.39	0.20
Ala	GCT	6.00	13.04	0.15
Ala	GCC	14.00	30.43	0.34
Arg	AGG	9.00	19.57	0.24
Arg	AGA	11.00	23.91	0.30
Ser	AGT	2.00	4.35	0.05
Ser	AGC	11.00	23.91	0.26
Lys	AAG	13.00	28.26	0.68
Lys	AAA	6.00	13.04	0.32
Asn	AAT	0.00	0.00	0.00
Asn	AAC	12.00	26.09	1.00
Met	ATG	9.00	19.57	1.00
Ile	ATA	1.00	2.17	0.08
Ile	ATT	2.00	4.35	0.15
Ile	ATC	10.00	21.74	0.77
Thr	ACG	5.00	10.87	0.28
Thr	ACA	2.00	4.35	0.11
Thr	ACT	2.00	4.35	0.11
Thr	ACC	9.00	19.57	0.50
Trp	TGG	8.00	17.39	1.00
End	TGA	1.00	2.17	1.00
Cys	TGT	1.00	2.17	0.12
Cys	TGC	7.00	15.22	0.88
End	TAG	0.00	0.00	0.00
End	TAA	0.00	0.00	0.00
Tyr	TAT	1.00	2.17	0.12
Tyr	TAC	7.00	15.22	0.88
Leu	TTG	3.00	6.52	0.08
Leu	TTA	1.00	2.17	0.03

Phe	TTT	6.00	13.04	0.25
Phe	TTC	18.00	39.13	0.75
Ser	TCG	11.00	23.91	0.26
Ser	TCA	4.00	8.70	0.09
Ser	TCT	6.00	13.04	0.14
Ser	TCC	9.00	19.57	0.21
Arg	CGG	4.00	8.70	0.11
Arg	CGA	4.00	8.70	0.11
Arg	CGT	0.00	0.00	0.00
Arg	CGC	9.00	19.57	0.24
Gln	CAG	11.00	23.91	0.73
Gln	CAA	4.00	8.70	0.27
His	CAT	0.00	0.00	0.00
His	CAC	6.00	13.04	1.00
Leu	CTG	9.00	19.57	0.24
Leu	CTA	4.00	8.70	0.11
Leu	CTT	4.00	8.70	0.11
Leu	CTC	17.00	36.96	0.45
Pro	CCG	8.00	17.39	0.29
Pro	CCA	7.00	15.22	0.25
Pro	CCT	5.00	10.87	0.18
Pro	CCC	8.00	17.39	0.29

Other modifications can also be made to the nucleotide sequence of interest to enhance its expression in duckweed. These modifications include, but are not limited to, elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well characterized sequences which may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence may be modified to avoid predicted hairpin secondary mRNA structures.

There are known differences between the optimal translation initiation context nucleotide sequences for translation initiation codons in animals and plants and the composition of these translation initiation context nucleotide sequence can influence the efficiency of translation initiation. See, for example, Lukaszewicz *et al.* (2000) *Plant Science* 154:89-98; and Joshi *et al.* (1997); *Plant Mol. Biol.* 35:993-1001. In the present invention, the translation initiation context nucleotide sequence for the translation initiation codon of the nucleotide sequence of interest may be modified to

enhance expression in duckweed. In one embodiment, the nucleotide sequence is modified such that the three nucleotides directly upstream of the translation initiation codon of the nucleotide sequence of interest are "ACC." In a second embodiment, these nucleotides are "ACA."

Expression of a transgene in duckweed can also be enhanced by the use of 5' leader sequences. Such leader sequences can act to enhance translation. One or more leader sequences may be used in combination to enhance expression of the target nucleotide sequence. Translation leaders are known in the art and include, but are not limited to, picornavirus leaders, e.g., EMCV leader (Encephalomyocarditis 5' noncoding region; Elroy-Stein *et al.* (1989) *Proc. Natl. Acad. Sci USA* 86:6126); potyvirus leaders, e.g., TEV leader (Tobacco Etch Virus; Allison *et al.* (1986) *Virology* 154:9); human immunoglobulin heavy-chain binding protein (BiP; Macajak and Sarnow (1991) *Nature* 353:90); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4; Jobling and Gehrke (1987) *Nature* 325:622); tobacco mosaic virus leader (TMV; Gallie (1989) *Molecular Biology of RNA*, 23:56); potato etch virus leader (Tomashevskaya *et al.* (1993) *J. Gen. Virol.* 74:2717-2724); Fed-1 5' untranslated region (Dickey (1992) *EMBO J.* 11:2311-2317); RbcS 5' untranslated region (Silverthorne *et al.* (1990) *J. Plant. Mol. Biol.* 15:49-58); and maize chlorotic mottle virus leader (MCMV; Lommel *et al.* (1991) Virology 81:382). See also, Della-Cioppa *et al.* (1987) *Plant Physiology* 84:965. Leader sequence comprising plant intron sequence, including intron sequence from the maize dehydrogenase 1 gene, the castor bean catalase gene, or the Arabidopsis tryptophan pathway gene PAT1 has also been shown to increase translational efficient in plants (Callis *et al.* (1987) *Genes Dev.* 1:1183-1200; Mascarenhas *et al.* (1990) *Plant Mol. Biol.* 15:913-920). In one embodiment of the present invention, nucleotide sequence corresponding to nucleotides 1222-1775 of the maize alcohol dehydrogenase 1 gene (GenBank Accession Number X04049), set forth in SEQ ID NO: 1, is inserted upstream of the nucleotide sequence of interest to enhance the efficiency of its translation. In another embodiment, the expression vector contains the leader from the *Lemna gibba* ribulose-bis-phosphate carboxylase small subunit 5B gene (Buzby *et al.* (1990) *Plant Cell* 2:805-814).

It is recognized that any of the duckweed expression-enhancing nucleotide sequence modifications described above can be used in the present invention, including any single modification or any possible combination of modifications. The phrase "modified for enhanced expression in duckweed" as used herein refers to a nucleotide sequence that contains any one or any combination of these modifications.

D. Signal Peptides

Secreted proteins are usually translated from precursor polypeptides that include a "signal peptide" that interacts with a receptor protein on the membrane of the endoplasmic reticulum (ER) to direct the translocation of the growing polypeptide chain across the membrane and into the endoplasmic reticulum for secretion from the cell. This signal peptide is often cleaved from the precursor polypeptide to produce a "mature" polypeptide lacking the signal peptide. In an embodiment of the present invention, a biologically active polypeptide is expressed in duckweed from a nucleotide sequence that is operably linked with a nucleotide sequence encoding a signal peptide that directs secretion of the polypeptide into the culture medium. Plant signal peptides that target protein translocation to the endoplasmic reticulum (for secretion outside of the cell) are known in the art. See, for example, U.S. Patent No. 6,020,169 to Lee *et al.* In the present invention, any plant signal peptide can be used to in target polypeptide expression to the ER. In some embodiments, the signal peptide is the *Arabidopsis thaliana* basic endochitinase signal peptide, the extensin signal peptide (Stiefel *et al.* (1990) *Plant Cell* 2:785-793), or the rice α-amylase signal peptide (SEQ ID NO:8; amino acids 1-31 of NCBI Protein Accession No. AAA33885). In another embodiment, the signal peptide corresponds to the signal peptide of a secreted duckweed protein.

Alternatively, a mammalian signal peptide can be used to target recombinant polypeptides expressed in genetically engineered duckweed for secretion. It has been demonstrated that plant cells recognize mammalian signal peptides that target the endoplasmic reticulum, and that these signal peptides can direct the secretion of polypeptides not only through the plasma membrane but also through the plant cell wall. See U.S. Patent Nos. 5,202,422 and 5,639,947 to Hiatt *et al.*

In some embodiment, the nucleotide sequence encoding the signal peptide is modified for enhanced expression in duckweed, utilizing any modification or combination of modifications disclosed in section B above for the nucleotide sequence of interest. For example, a duckweed optimized sequence encoding the signal peptide from rice α-amylase is shown in SEQ ID NO:7. This sequence contains approximately 93% duckweed preferred codons.

The secreted polypeptide can be harvested from the culture medium by any conventional means known in the art and purified by chromatography, electrophoresis, dialysis, solvent-solvent extraction, and the like.

E. Transformed Duckweed Plants and Duckweed Nodule Cultures

The stably transformed duckweed utilized in this invention can be obtained by any method known in the art. In one embodiment, the stably transformed duckweed is obtained by one of the gene transfer methods disclosed in U.S. Patent No. 6,040,498 or U.S. Patent Publication Numbers 20030115640, 20030033630, or 20020088027; each of which is herein incorporated by reference. These methods include gene transfer by ballistic bombardment with microprojectiles coated with a nucleic acid comprising the nucleotide sequence of interest, gene transfer by electroporation, and gene transfer mediated by *Agrobacterium* comprising a vector comprising the nucleotide sequence of interest. In some embodiments, the stably transformed duckweed is obtained via any one of the *Agrobacterium*-mediated methods disclosed in U.S. Patent No. 6,040,498 to Stomp *et al.* The *Agrobacterium* used is *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*.

Stably transformed duckweed plants may also be obtained by chloroplast transformation. See, for example, U.S. provisional patent application number 60/492,179, filed August 1, 2003, entitled "Chloroplast transformation of duckweed." Stably transformed duckweed lines may also be produced using plant virus expression vectors. See, for example, U.S. Patent Number 6,632,980 and Koprowski and Yusibov (2001) *Vaccine* 19:2735-2741.

It is preferred that the stably transformed duckweed plants utilized in these methods exhibit normal morphology and are fertile by sexual reproduction. Preferably, transformed plants of the present invention contain a single copy of the

transferred nucleic acid, and the transferred nucleic acid has no notable rearrangements therein. Also preferred are duckweed plants in which the transferred nucleic acid is present in low copy numbers (i.e., no more than twelve copies, no more than eight copies, no more than five copies, alternately, no more than three copies, as a further alternative, fewer than three copies of the nucleic acid per transformed cell).

EXPERIMENTAL

The following examples are offered for purposes of illustration, not by way of limitation.

Expression Constructs for the Production of Plasminogen and Microplasminogen in Duckweed

The expression vector used in the present examples is a modified version of pBMSP-1, which is described in U.S. Patent No. 5,955,646, herein incorporated by reference. The transcriptional cassette of the vector contained three copies of a transcriptional activating nucleotide sequence derived from the *Agrobacterium tumefaciens* octopine synthase and, an additional transcriptional activating nucleotide sequence derived from the *Agrobacterium tumefaciens* mannopine synthase gene, a promoter region derived from the *Agrobacterium tumefaciens* mannopine synthase gene, a polylinker site for insertion of the nucleotide sequence encoding the polypeptide of interest, and a termination sequence derived from the *Agrobacterium tumefaciens* nopaline synthase gene (see, van Engelen *et al.* (1995) 4:288-290; Ni *et al.* (1995) *Plant J.* 7:661-76; and Luehrsen *et al.* (1991) *Mol. Gen. Genet.* 225:81-93, each of which is herein incorporated by reference).

The expression vector also contained a nucleotide sequence coding for gentamycin acetyltransferase-3-I, aacC1, Wohlleben *et al.* (1989) *Mol. Gen. Genet.* 217:202-208) encoding gentamycin resistance as a selectable marker. Transcription of the selectable marker sequence is driven by a promoter derived from the *Agrobacterium tumefaciens* nopaline synthase II gene.

The expression vector additionally contains the leader from the ribulose-bis-phosphate carboxylase small subunit 5B gene of *Lemna gibba* (nucleotides 689-751 of NCBI Accession No. S45167, Buzby *et al.* (1990) *Plant Cell* 2:805-814) and a

nucleotide sequence corresponding to nucleotides 1222-1775 of the maize alcohol dehydrogenase gene (GenBank Accession Number X04049) inserted between the promoter and the polylinker. This intron sequence is shown in SEQ ID NO:1, and the leader sequence is shown in SEQ ID NO:2.

Transformation of Duckweed

Duckweed fronds or duckweed nodule cultures (derived from *Lemna minor* strain 8627 in these examples) were transformed with the expression constructs described above using Agrobacterium-mediated transformation methods. *Agrobacterium tumefaciens* strain C58Z707, a disarmed, broad host range C58 strain (Hepburn *et al.* (1985) *J. Gen. Microbiol. 131*:2961-2969) is used for transformation in these examples. The expression constructs described above were mobilized into *A. tumefaciens* by electroporation, or by a triparental mating procedure using *E. coli* MM294 harboring the mobilizing plasmid pRK2013 (Hoekema *et al.* (1983) *Nature 303*: 179-180; Ditta *et al.* (1980) *Proc. Natl. Acad. Sci. USA 77*: 7347-7350). C58Z707 strains comprising the expression constructs described above are streaked on AB minimal medium (Chilton *et al.*, (1974) *Proc. Nat. Acad. Sci. USA 71*: 3672 - 3676) or in YEB or LB medium (1 g/L yeast extract, 5 g/L beef extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L $MgSO_4$) containing streptomycin at 500 mg/L, spectinomycin at 50 mg/L and kanamycin sulfate at 50 mg/L and grown overnight at 28° C.

Duckweed nodule cultures for transformation were produced as follows. Duckweed fronds were separated, the roots are cut off with a sterile scalpel, and the fronds are placed, ventral side down, on Murashige and Skoog medium (catalog number M-5519; Sigma Chemical Corporation, St. Louis, MO) pH 5.6, supplemented with 5 μM 2,4-dichlorophenoxyacetic acid, 0.5 μM 1-Phenyl-3(1,2,3-thiadiazol-5-yl) urea thidiazuron (Sigma P6186), 3% sucrose, 0.4 Difco Bacto-agar (Fisher Scientific), and 0.15% Gelrite (Sigma). Fronds were grown for 5-6 weeks. At this time, the nodules (small, yellowish cell masses) appeared, generally from the central part of the ventral side. This nodule tissue was detached from the mother frond and cultured in Murashige and Skoog medium supplemented with 3% sucrose, 0.4% Difco Bacto-agar, 0.15% Gelrite, 1 μM 2,4-dichlorophenoxyacetic acid, and 2 μM benzyladenine.

Duckweed nodule cultures were transformed as follows. The appropriate *Agrobacterium tumefaciens* strain was grown on potato dextrose agar or YEB or LB agar with 50 mg/L kanamycin and 100 μM acetosyringone, and resuspended in Murashige and Skoog medium supplemented with 0.6 M Mannitol and 100 μM acetosyringone. Nodule culture tissue was inoculated by immersing in the solution of resuspended bacteria for 1-2 minutes, blotted to remove excess fluid, and plated on co-cultivation medium consisting of Murashige and Skoog medium supplemented with auxin and cytokinin optimized to promote nodule growth and 100 μM acetosyringone. *See*, Yamamoto *et al.* (2001) *In Vitro Cell Dev. Biol. Plant* 37:349-353.

For selection, nodule culture tissue was transferred to regeneration medium; 0.5 X Schenk and Hildebrandt medium supplemented with 1% sucrose 0.4 % Difco Bacto-Agar, 0.15% Gelrite 500 mg/L cefotaxime, and 6 mg/L geneticin and cultured for approximately 6-8 weeks under continuous light (20-40 $\mu M/m^2 \cdot sec$). The nodule tissue was transferred every 7 days to fresh culture medium. Selection is complete when the nodule tissue shows vigorous growth on the selection agent.

The following examples demonstrate the expression of biologically active plasminogen and microplasminogen in duckweed.

Example 1: Production of Plasminogen

Human plasminogen was expressed in duckweed as follows. A synthetic duckweed-codon optimized sequence encoding human plasminogen was inserted into the expression vector described above. The amino acid sequence of the encoded plasminogen is given in SEQ ID NO:4, and the duckweed-optimized coding sequence is given in SEQ ID NO:3. The expression vector also contained a duckweed optimized coding sequence encoding the rice alpha amylase signal peptide inserted 5' of the plasminogen coding sequence. The nucleotide sequence encoding the signal peptide was operably linked to the nucleotide sequence encoding human plasminogen such that the two coding sequences would be translated as one protein. The duckweed optimized coding sequence encoding the rice alpha amylase signal peptide

is given in SEQ ID NO:7, and the encoded peptide is given in SEQ ID NO:8. This plasminogen expression vector is referred to as BAP01 in this example.

Duckweed lines transformed with the BAP01 expression vector were generated as described above. Due to the large size of plasminogen, the primary screening of transgenic lines included analysis of both media and tissue homogenates. The vast majority of expressed protein was retained in the tissue. A total of 81 lines were generated and plasminogen accounted for as much as 6% of the total soluble protein in plant extracts (as measured by ELISA). Figure 1 represents the level of plasminogen measured in 56 of the transgenic lines. These levels were obtained from plants grown for 2 weeks in research vessels. Commercially available plasminogen was used as the standard in this assay.

The activity of the duckweed-expressed plasminogen was determined for tissue extracts from 7 different independent transgenic lines. The duckweed-expressed plasminogen was activated by streptokinase to produce an active complex. Activation by streptokinase does not involve the formation of plasmin, but occurs by a conformational shift that results from formation of a streptokinase/plasminogen complex. The activity of the resulting complex was then determined by assaying for cleavage of the chromogenic substrate Glu-Phe-Lys-pNA (available from Chromgenix Instrumentation Laboratory SpA, Milano Italy) shown in SEQ ID NO:9 (pNA=p-Nitroanilide) at 405 nm. (Gram J. and Jespersen J. Thromb. Haemost. 53, 255-259 (1985) and Robbins, K.C. Semin. Thromb. Haemost. 13 (2), 131-138 (1987)). For each of the seven transgenic lines tested, the activity level was closely correlated with the protein expression levels as determined by ELISA, indicating that the plasminogen produced in *Lemna* had a similar specific activity to that of the commercially available control protein.

It was noted that many of the duckweed lines that were engineered to over express plasminogen underwent rapid senescence. However, it was determined that senescence in these lines could be reduced by increasing the aeration and media volume during plant culture. In addition, altering the inoculum density can also influence the plant health. The culture of eight transgenic duckweed lines was scaled-up, and one of these lines, BAP01-B1-95, was selected for further analysis. This line was selected based on biomass accumulation, plasminogen expression level (3.3% of

total soluble protein levels in crude plant extracts as determined by ELISA), and overall plant health.

Plasminogen was harvested from the BAP01-B1-95 line as follows. Bulk duckweed tissue was homogenized, clarified by centrifugation, filtered through a 0.22 μM filter, passed through a Dowex ion exchange resin column (available from Dow Chemical, Midland, Mich.), and then affinity purified by lysine Sepharose chromatography. Bound material was eluted from the affinity column with ϵ-amino caproic acid. Crude tissue extracts obtained from these plants contained plasminogen as 3.3% of the total soluble protein as measured by ELISA.

The activity of the duckweed-produced plasminogen following activation by tPA urokinase, and streptokinase was also determined. Activity of plasminogen activated with either tPA or urokinase produced activated plasmin as shown in Figure 2. Western blotting analysis showed that both the heavy and light chain of the plasmin produced by activation the duckweed-produced plasminogen co-migrated with the commercially available plasmin used as a control (Figure 6). Activation with streptokinase also produced activated plasminogen as shown in SEQ ID NO:3. In this assay, plasminogen is activated by streptokinase to produce an active complex, which then cleaves a chromogenic substrate (Coamatic® brand plasminogen kit, DiaPharma, West Chester, OH). Figure 3 shows that for the lines tested, quantitation by ELISA and streptokinase activity assay gave comparable values, indicating that *Lemna*-produced plasminogen has a similar specific activity to the control protein.

The size of the duckweed-produced plasminogen was determined by Western blotting using an anti-plasminogen antibody available from American Diagnostica, Inc. Greenwich, CT. This analysis showed that 60% of the plasminogen produced in duckweed was full length N-terminal sequencing of the duckweed produced plasminogen showed further processing producing a polypeptide missing the first 74 amino acids. In human serum, plasminogen is isolated as a mixture of unprocessed 'glu-plasminogen' and several processed versions defined as 'lys-plasminogen' where 69, 77, or 78 N-terminal amino acids are removed. Such a cleavage has no effect on activity.

Because references in the prior art have reported difficulty in producing stable plasminogen in recombinant systems, the stability of plasminogen produced in the

Lemna system was tested. Figure 4 demonstrates the stability of human plasminogen added to a *Lemna* tissue extract. The figure demonstrates that the human plasminogen retained almost all of its activity even after an overnight incubation in the *Lemna* extract. Figure 5 demonstrates the stability of *Lemna*-produced plasminogen in *Lemna* tissue extracts following a freeze-thaw cycle. The figure demonstrates that the *Lemna*-produced protein is stable following a freeze-thaw cycle.

Example 2: Production of Microplasminogen in Duckweed

Human microplasminogen was expressed in duckweed as follows. A synthetic duckweed-codon optimized sequence encoding human microplasminogen was inserted into the expression vector described above. The amino acid sequence of the encoded microplasminogen is given in SEQ ID NO:6, and the duckweed-optimized coding sequence is given in SEQ ID NO:5. The expression vector also contained a duckweed optimized coding sequence encoding the rice alpha amylase signal peptide inserted 5' of the plasminogen coding sequence. The nucleotide sequence encoding the signal peptide was operably linked to the nucleotide sequence encoding human microplasminogen such that the two coding sequences would be translated as one protein. The duckweed optimized coding sequence encoding the rice alpha amylase signal peptide is given in SEQ ID NO:7, and the encoded peptide is given in SEQ ID NO:8. This microplasminogen expression vector is referred to as BAMP01 in this example.

Duckweed lines transformed with the BAMP01 expression vector were generated as described above. The majority of the expressed microplasminogen was secreted into the culture medium. 79 transgenic lines were screened to determine microplasminogen expression levels. Figure 7 shows the levels of microplasminogen expressed in these lines. The first set of 42 lines were grown for 7 days, while the second set of 37 lines were grown for six days. It should be noted that duckweed is unique in that it grows in very dilute aqueous media inorganic media with a very low protein content, distinguishing it from type fermentation based systems and mammalian cell-based expression systems. The duckweed growth media typically contains only 30 mg/L of host plant proteins. This low level of host plant protein provides advantages for the downstream purification of the secreted protein.

One of the BAMP01 transformed duckweed lines, BAMP01-B1-58, was selected for further study. Microplasminogen was harvested from the culture media of the BAMP01-B1-58 line as follows. The culture media was process by ultrafiltration and diafiltration and then passed over a Dowex ion exchange resin column (available from Dow Chemical, Midland, Mich.) to remove low molecular weight plant metabolites. The concentration before concentrating of microplasminogen in the crude aqueous media was approximately 20 mg/L as determined by quantitative Western blotting.

The size of the duckweed-produced microplasminogen was determined by Western blotting using an anti plasminogen antibody available from (American Diagnostica Inc. Greenwich, CT). This analysis showed that most of the microplasminogen produced in duckweed was full length. An intact N-terminus was confirmed also by N-terminal sequencing

The activity of the duckweed-expressed microplasminogen was determined for the BAMP01-B1-58 transgenic line following activation by streptokinase to produce an active complex as described above for plasminogen. The duckweed-produced microplasminogen showed some level of activity in the absence of streptokinase, and a significant increase in activity following activation by streptokinase.

Like plasminogen, microplasminogen can be activated by urokinase and tPA; however, only the B-chain is produced. Western blot analysis confirmed the production of the B chain of plasmin following activation of duckweed-produced microplasminogen with either urokinase or tPA.

To confirm the activity of plasmin from *Lemna*-produced microplasminogen, a gelatin zymogram was run on concentrated media following activation by tPA. Figure 8 shows the presence of an active proteolytic band that is not present in concentrated control media.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended embodiments.

WHAT IS CLAIMED IS:

1. A method for producing plasminogen in duckweed, wherein said method comprises the steps of:

(a) culturing a duckweed plant or duckweed plant cell or nodule, wherein said duckweed plant or duckweed plant cell or nodule is stably transformed with a nucleic acid molecule comprising a nucleotide sequence encoding plasminogen; and

(b) collecting said plasminogen from said duckweed plant or duckweed plant cell or nodule.

2. The method of claim 1, wherein said nucleotide sequence encoding plasminogen is operably linked to a coding sequence for a signal peptide that directs secretion of the microplasminogen into the culture medium.

3. The method of claim 1, wherein said nucleotide sequence encoding plasminogen has at least one attribute selected from the group consisting of:

(a) duckweed-preferred codons in the coding sequence for said plasminogen;

(b) an operably linked nucleotide sequence comprising a plant intron that is inserted upstream of the coding sequence; and

(c) an operably linked nucleotide sequence comprising a leader sequence that increases the translation of said nucleotide sequence encoding plasminogen.

4. The method according to claim 3, wherein the nucleotide sequence encoding plasminogen comprises between 70-100 % duckweed-preferred codons.

5. The method according to claim 3, wherein said plant intron is derived from the maize alcohol dehydrogenase 1 gene.

6. The method according to claim 5, wherein said plant intron consists essentially of the sequence set forth in SEQ ID NO:1.

7. The method according to claim 3, wherein said leader sequence is derived from the ribulose-bis-phosphate carboxylase small subunit 5B gene of *Lemna gibba*.

8. The method according to claim 7, wherein said leader sequence consists essentially of the nucleotide sequence shown in SEQ ID NO:2.

9. The method according to claim 3, wherein said nucleotide sequence encoding plasminogen is the nucleotide sequence shown in SEQ ID NO:3.

10. The method according to claim 1, wherein said plasminogen is human plasminogen.

11. The method according to claim 10, wherein said plasminogen has at least 95% sequence identity with the amino acid sequence set forth in SEQ ID NO:4.

12. The method according to claim 1, wherein at least 2% of the soluble protein in the duckweed plant or duckweed plant cell is plasminogen.

13. The method according to claim 12, wherein at least 3% of the soluble protein in the duckweed plant or duckweed plant cell is plasminogen.

14. The method according to claim 13, wherein at least 4% of the soluble protein in the duckweed plant or duckweed plant cell is plasminogen.

15. A method for producing microplasminogen in duckweed, said method comprising the steps of:

(a) culturing within a duckweed culture medium a duckweed plant culture, a duckweed plant cell, or a duckweed nodule culture, or wherein said

duckweed plant culture, plant cell, or nodule culture is stably transformed with a nucleic acid molecule comprising a nucleotide sequence encoding microplasminogen and an operably linked coding sequence for a signal peptide that directs secretion of the microplasminogen into the culture medium; and

(b) collecting said microplasminogen from the duckweed culture medium.

16. The method of claim 15, wherein said microplasminogen is secreted into the duckweed culture medium.

17. The method of claim 16, wherein said duckweed culture medium contains at least 1 mg/L microplasminogen as determined by quantitative Western blotting.

18. The method of claim 16, wherein said duckweed culture medium contains at least 2 mg/L microplasminogen as determined by quantitative Western blotting.

19. The method of claim 16, wherein said duckweed culture medium contains at least 5 mg/L microplasminogen as determined by quantitative Western blotting.

20. The method of claim 19, wherein said duckweed culture medium contains at least 10 mg/L microplasminogen as determined by quantitative Western blotting.

21. The method of claim 15, wherein said nucleotide sequence encoding microplasminogen and an operably linked coding sequence for a signal peptide has at least one attribute selected from the group consisting of:

(a) duckweed-preferred codons in the coding sequence for said microplasminogen;

(b) duckweed-preferred codons in the coding sequence for said signal peptide;

(c) an operably linked nucleotide sequence comprising a plant intron that is inserted upstream of the coding sequence for microplasminogen; and

(d) an operably linked nucleotide sequence comprising a leader sequence that increases the translation of said nucleotide sequence encoding plasminogen.

22. The method according to claim 21, wherein the nucleotide sequence encoding plasminogen comprises between 70-100 % duckweed-preferred codons.

23. The method according to claim 22, wherein the nucleotide sequence encoding signal peptide comprises between 70-100 % duckweed-preferred codons.

24. The method according to claim 21, wherein said plant intron is derived from the maize alcohol dehydrogenase 1 gene.

25. The method according to claim 24, wherein said plant intron consists essentially of the sequence set forth in SEQ ID NO:1.

26. The method according to claim 21, wherein said leader sequence is derived from the ribulose-bis-phosphate carboxylase small subunit 5B gene of *Lemna gibba*.

27. The method according to claim 26, wherein said leader sequence consists essentially of the nucleotide sequence shown in SEQ ID NO:2.

28. The method according to claim 21, wherein said nucleotide sequence encoding microplasminogen is the nucleotide sequence shown in SEQ ID NO:5.

29. The method according to claim 15, wherein said microplasminogen is human microplasminogen.

30. The method according to claim 29, wherein said microplasminogen has at least 95% sequence identity with the amino acid sequence set forth in SEQ ID NO:4.

31. The method according to claim 15, wherein said signal peptide is the rice alpha amylase polypeptide.

32. The method according to claim 31, wherein the coding sequence for the signal peptide comprises the nucleotide sequence set forth in SEQ ID NO:7.

33. The method according to claim 15, wherein said signal peptide sequence has the amino acid sequence set forth in SEQ ID NO:8.

34. The method according to claim 1, wherein said duckweed plant or duckweed plant cell is from a genus selected from the group consisting of the genus *Spirodela*, genus *Wolffia*, genus *Wolfiella*, genus *Landoltia* and genus *Lemna*.

35. The method according to claim 15, wherein said duckweed plant or duckweed plant cell belongs to a genus selected from the group consisting of the genus *Spirodela*, genus *Wolffia*, genus *Wolfiella*, genus *Landoltia* and genus *Lemna*.

36. An improvement in a method of producing stable plasminogen, the improvement comprising producing the plasminogen according to the method of claim 1.

37. A method for producing a plasminogen fragment in duckweed, wherein said method comprises the steps of:

(a) culturing within a duckweed culture medium a duckweed plant or duckweed plant cell or nodule, wherein said duckweed plant or duckweed plant cell or nodule is stably transformed with a nucleic acid molecule comprising a nucleotide sequence encoding a plasminogen fragment; and

(b) collecting said plasminogen fragment from at least one source selected from said duckweed plant, said duckweed plant cell, said duckweed nodule, or said duckweed culture medium.

38. The method of claim 37, wherein said nucleotide sequence encoding the plasminogen fragment is operably linked to a coding sequence for a signal peptide that directs secretion of the microplasminogen into the culture medium.

39. The method of claim 37, wherein said nucleotide sequence encoding the plasminogen fragment has at least one attribute selected from the group consisting of:

(a) duckweed-preferred codons in the coding sequence for said plasminogen fragment;

(b) an operably linked nucleotide sequence comprising a plant intron that is inserted upstream of the coding sequence; and

(c) an operably linked nucleotide sequence comprising a leader sequence that increases the translation of said nucleotide sequence encoding the plasminogen fragment.

40. The method according to claim 39, wherein the nucleotide sequence encoding the plasminogen fragment comprises between 70-100 % duckweed-preferred codons.

41. The method according to claim 39, wherein said plant intron is derived from the maize alcohol dehydrogenase 1 gene.

42. The method according to claim 37, wherein said plasminogen fragment comprises at least 80 contiguous amino acids of a mature plasminogen amino acid sequence.

43. A stably transformed duckweed plant, duckweed plant cell, or nodule according to claim 1.

44. The stably transformed duckweed plant, duckweed plant cell, or nodule according to claim 43, wherein said duckweed plant, duckweed plant cell, or duckweed nodule is from a genus selected from the group consisting of the genus *Spirodela*, genus *Wolffia*, genus *Wolfiella*, genus *Landoltia* and genus *Lemna*.

45. The stably transformed duckweed plant, duckweed plant cell or duckweed nodule according to claim 44, wherein said duckweed plant, duckweed plant cell, or duckweed nodule is a member of a species selected from the group consisting of *Lemna minor*, *Lemna miniscula*, *Lemna aequinoctialis*, and *Lemna gibba*.

46. A stably transformed duckweed plant, duckweed plant cell, or duckweed nodule according to claim 15.

47. The stably transformed duckweed plant, duckweed plant cell, or duckweed nodule according to claim 46, wherein said duckweed plant, duckweed plant cell, or duckweed nodule is from a genus selected from the group consisting of the genus *Spirodela*, genus *Wolffia*, genus *Wolfiella*, genus *Landoltia* and genus *Lemna*.

48. The stably transformed duckweed plant or duckweed plant cell according to claim 47, wherein said duckweed plant or duckweed nodule is a member of a species selected from the group consisting of *Lemna minor*, *Lemna miniscula*, *Lemna aequinoctialis*, and *Lemna gibba*.

49. A stably transformed duckweed plant, duckweed plant cell, or duckweed nodule according to claim 37.

50. The stably transformed duckweed plant, duckweed plant cell, or duckweed nodule according to claim 49, wherein said duckweed plant, duckweed plant cell, or duckweed nodule is from a genus selected from the group consisting of

the genus *Spirodela*, genus *Wolffia*, genus *Wolfiella*, genus *Landoltia* and genus *Lemna*.

51. The stably transformed duckweed plant or duckweed plant cell according to claim 50, wherein said duckweed plant or duckweed nodule is a member of a species selected from the group consisting of *Lemna minor*, *Lemna miniscula*, *Lemna aequinoctialis*, and *Lemna gibba*.

52. An isolated nucleic acid molecule comprising a nucleotide sequence encoding an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence set forth in SEQ ID NO:4;

(b) the amino acid sequence of a variant of the plasminogen sequence shown in SEQ ID NO:4, wherein said variant has at least 95% sequence identity with the amino acid sequence set forth in SEQ ID NO:6;

(c) the amino acid sequence set forth in SEQ ID NO:6; and

(d) the amino acid sequence of a variant of the microplasminogen sequence shown in SEQ ID NO:6, wherein said variant has at least 95% sequence identity with the amino acid sequence set forth in SEQ ID NO:6;

wherein said nucleotide sequence comprises between 70-100% duckweed-preferred codons.

53. The nucleic acid molecule of claim 49, wherein said nucleic acid molecule comprises a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in SEQ ID NO:3; and

(b) the nucleotide sequence set forth in SEQ ID NO:5.

54. A stably transformed duckweed plant, wherein said duckweed comprises a DNA construct containing the following operably linked elements, a leader sequence, a promoter sequence, a nucleotide sequence encoding plasminogen, and a transcriptional termination sequence, wherein said leader sequence, said promoter sequence and said termination sequence all function in duckweed.

55. The plant of claim 54, wherein said leader sequence is the sequence set forth in SEQ ID NO:2 and the sequence encoding plasminogen is set forth in SEQ ID NO:3.