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(54) Title: THE FLU GENE: A TOOL FOR THE IDENTIFICATION OF GENES INVOLVED IN STRESS RESPONSES AND APOPTOSIS AND A TARGET FOR HERBICIDES SPECIFIC FOR ANGIOSPERMS

(57) Abstract: The present invention relates to new target genes for herbizides specific for angiosperms. Furthermore, the invention relates to modified transgenes that confer resistance to said herbizides. The new target genes in combination with the herbizide of the invention are applied as selectable markers. Furthermore, the present invention is concerned with the indentification of genes that form part of signal transduction pathways.

The *FLU* gene: A tool for the identification of genes involved in stress responses and apoptosis and a target for herbicides specific for angiosperms

The present invention is based on the identification of the *FLU* gene that plays a key role in the regulation of tetrapyrrole biosynthesis of angiosperms. The present invention is generally concerned with the exploitation of the *flu* mutant for the identification of genes that form part of signal transduction pathways involved in triggering various stress responses and apoptosis. More particularly, the invention relates to the identification of new genes and their products that can be exploited as potential targets for compounds that display antiapoptotic activities and enhance the tolerance of plants to biotic and abiotic stressors. Furthermore, the invention relates to the use of *FLU* and its product as a target for herbicides specific for angiosperms and the production and application of new and more specific herbicides based thereon. The *FLU* gene in combination with the herbicide is applied as a selectable marker. Finally, the invention relates to a modified *FLU* transgene that confers resistance to said herbicides.

Background of the invention

Tetrapyrroles such as chlorophylls and bacteriochlorophylls play a fundamental role in the energy absorption and transduction activities of photosynthetic organisms. Because of these molecules, however, photosynthetic organisms are also prone to photooxidative damage. They had to evolve highly efficient strategies to control tetrapyrrole biosynthesis and to prevent the accumulation of free intermediates that potentially may act as photosensitizers and upon illumination may lead to the release of reactive

oxygen species (ROS). In higher plants, the metabolic flow of tetrapyrrole biosynthesis is regulated at the step of δ-aminolevulinic acid synthesis. This regulation previously has been attributed to feedback control of Glu tRNA reductase, the first enzyme committed to tetrapyrrole biosynthesis, by heme (v. Wettstein et al., 1995).

In plants ROS are continuously produced as byproducts of various metabolic pathways that are localized in different cellular compartments (Foyer and Harbinson, 1994). Under physiological steady state conditions these molecules are scavenged by different antioxidative defense components (Alscher et al., 1997). The equilibrium between production and scavenging of ROS may be perturbed by a number of adverse environmental factors. As a result of these disturbances, intracellular levels of ROS may rapidly rise (Malan et al., 1990; Elstner, 1991; Prasad et al., 1994; Orozco-Cardenas and Ryan, 1999; Tsugane et al., 1999). The rapid increase in ROS concentration has been named "oxidative burst" (Apostol et al., 1989). Such an oxidative burst may have several immediate consequences; ROS may directly kill plant cells or intruding pathogens and it may contribute to the reinforcement of cell walls (Levine et al., 1994). ROS have also been implicated as second messengers controlling stress responses (Dangl et al., 1996; Low and Merida, 1996; Kovtun et al., 2000) and have consequently been proposed to either diffuse into neighboring leaf areas and induce directly defense reactions, or to activate at the site of the applied stress stimulus a signal transduction pathway that controls defense reactions in other leaf areas (Karpinski et al., 1999). In this way ROS could induce the synthesis of antioxidative enzymes and components that increase the overall scavenging capacity for ROS and help to readjust their concentrations to their initial low steady state levels

(Karpinski et al., 1997; 1999). At the same time they might also trigger additional defense responses that permit the plant to withstand the stress (Malan et al., 1990; Wu et al., 1995; Tsugane et al., 1999; Bowler and Fluhr, 2000).

Even though most studies agree on the importance of ROS as a mediator of stress defense reactions and apoptosis, not many details are known of how the increase of ROS concentration is perceived by the plant and how this information is translated into signals that direct the plant's response to stress. This lack of knowledge has several reasons. In many cases, it is not clear whether the biological activity of ROS is due to its direct toxic effect or reflects the more indirect role of a second messenger that signals further cellular responses, or both (Dangl et al., 1996). The term "ROS" embraces chemically distinct oxygen derivatives that may be produced selectively by a particular cellular compartment in response to a specific environmental cue. It is not known to what extent chemical specificity of these ROS species and the cellular topography of their release may contribute to the multiplicity of stress responses in plants.

In previous works several steps of tetrapyrrole biosynthesis had been blocked experimentally either by expressing antisense RNA in transgenic plants (Mock et al., 1998; Molina et al., 1999) or by treating plants with enzyme-specific inhibitors (Matringe et al., 1989). A broad range of stress-related reactions were activated in these plants during illumination that have been attributed to the photodynamic activity of free tetrapyrrole intermediates that accumulated constitutively in these plants (Molina et al., 1999; Mock et al., 1999). Similar effects of accumulated tetrapyrroles were also evident in mutants that carried lesions in genes

encoding the uroporphyrinogen decarboxylase (Hu et al., 1998) and the red Chl catabolite reductase (Mach et al., 2001) and in seedlings that were fed ALA (Rebeiz et al., 1988). The likely primary cause for the stress reactions in these plants is the light-dependent generation of singlet oxygen (Chakraborty and Tripathy, 1992; Boo et al., 2000). However, many aspects of the responses were reminiscent of those triggered by the release of superoxide and/or hydrogen peroxide during an incompatible plant-pathogen interaction (Mock et al., 1999; Molina et al., 1999). Either singlet oxygen, superoxide and hydrogen peroxide may replace each other in triggering pathogen defense reactions, or the constitutive accumulation of photodynamically active tetrapyrrole intermediates and catabolites throughout the entire life cycle of these genetically modified plants may lead to photooxidative damage and injury that may promote a multifactorial induction of several overlapping secondary effects, some of which may mimic responses induced by pathogens.

Summary of the invention

Up to now almost nothing is known about the molecular mechanism, that controls chlorophyll synthesis.
The present invention uses a genetic approach to identify a key element of the feedback control in Arabidopsis that operates independently of the heme-dependent circuit and selectively affects the magnesium branch of tetrapyrrole biosynthesis. First, mutants of *Arabidopsis thaliana* were identified that are no longer able to restrict the accumulation of Pchlide in the dark. Such mutants can easily be distinguished from wildtype seedlings by the strong Pchlide fluorescence that etiolated mutant seedlings emit

after they have been exposed to blue light (Fig. 1). Because of this trait, these mutants have been named *flu* (fluorescent). They resemble dark-grown wildtype seedlings that were fed with exogenous ALA (Granick, 1959) and etiolated *tigrina* mutants of barley (Nielsen, 1974, von Wettstein et al., 1995). Out of a total of 1'500 M2 seed families of EMS mutagenized Arabidopsis plants four independent *flu* mutants were identified, all of which segregated in a 1 : 3 ratio. Crosses between these recessive mutants revealed that they are allelic and hence represented a single gene. When etiolated seedlings of the homozygous *flu* mutant were transferred from the dark to the light, they rapidly bleached and died (Fig. 1). The homozygous mutant could be rescued, however, by germinating the seedlings under constant light (Fig. 1). Under these conditions Pchlide was continuously photoreduced to Chlide. Mutant plants grown under constant light were indistinguishable from wildtype and produced similar amounts of viable seeds.

If FLU forms part of a feedback loop that down-regulates ALA synthesis in dark-grown seedlings, its inactivation should result in an enhanced rate of ALA formation. As shown in Fig. 2a, the rate of ALA synthesis in the *flu* mutant does indeed exceed that of the wildtype by a factor of 3 to 4. Two different mechanisms have previously been considered to explain the regulation of ALA formation: Light-induced changes in the synthesis of enzymes required for its formation (Nadler and Granick, 1970) and the removal of an inhibitor affecting the activity of one of these enzymes (Fluhr et al., 1975). We have tested the former possibility by measuring the concentrations of mRNAs for the two enzymes committed exclusively to ALA synthesis: Glu tRNA reductase and Glu 1-semialdehyde aminotransferase (GSA). Drastic

light/dark fluctuations of these two mRNAs have been described earlier for Arabidopsis (Ilag et al., 1994). These changes in mRNA levels during a light/dark shift could form the basis for the restriction of ALA synthesis in the dark. In such a case the enhanced rate of ALA synthesis in *flu* mutants transferred to the dark could be caused by a constitutive upregulation of the two mRNAs. However, in the mutant the two transcripts showed the same fluctuations during a light to dark transition as the wildtype plants. Also in the *tigrina-d*^{12} mutant of barley that resembles closely the *flu* mutant of *Arabidopsis* accumulation of Pchlide in etiolated mutant seedlings could not be explained by a change in the concentrations of enzymes involved in ALA synthesis and their mRNAs (Hansson et al., 1997). Therefore, we favor the second model that predicts direct metabolic feedback inhibition of tetrapyrrole biosynthesis.

In analogy to its regulatory role in animals and yeast, heme has been proposed to act also in plants as an effector of feedback inhibition of tetrapyrrole biosynthesis (Beale and Weinstein, 1990). Several lines of evidence support this assumed function of heme. The activity of Glu tRNA reductase, the first enzyme committed to ALA synthesis and the most likely target of feedback control, has been shown to be inhibited *in vitro* by heme (Vothknecht et al., 1998). Inactivation of a heme oxygenase gene in the *hy1* mutant of Arabidopsis perturbs the breakdown of heme, attenuates the rate of ALA synthesis, and suppresses Pchlide accumulation in etiolated seedlings (Terry and Kendrick, 1999). Conversely, removal of free $Fe^{2+/3+}$ by the addition of an iron chelator leads to a decline of the heme level and causes an increase in the level of Pchlide (Duggan and Gassman, 1974). If FLU forms part of the heme-dependent feedback loop, its

inactivation in the *flu* mutant should not only enhance the level of Pchlide but also the level of free heme. However, in etiolated *flu* mutants only the level of Pchlide but not that of free heme was higher than in wildtype seedlings (Fig. 2b,c). Furthermore in double mutant lines of *hy1* and *flu* the Pchlide level was intermediate, approximately four to five times higher than in *hy1* but significantly lower than in *flu* (Fig. 3). *flu* antagonizes the inhibitory effect of elevated levels of heme in *hy1* and thus seems to act via a second independent regulatory circuit. In wildtype plants both heme and FLU seem to inhibit ALA synthesis and the actual metabolic flux of tetrapyrrole synthesis seems to be determined by the additive effects of both regulators.

When grown under continuous white light up to maturity no differences between wildtype and *flu* plants could be detected. However, once these plants were transferred to the dark, *flu* mutants started to accumulate minute amounts of Pchlide (Fig. 4). Once these plants were returned to the light several stress responses were induced in *flu*. In contrast to etiolated seedlings of *flu* that rapidly died during illumination, mature light-adapted *flu* plants at the rosette leaf stage did not show any immediate damages or stress responses. Only after 45 to 60 min the beginning of cell death could be documented by trypan blue staining of leaves and after 3 to 4 hours following reillumination visible necrotic lesions started to develop (Fig. 5). A second striking response of the *flu* mutant to the dark/light shift is the inhibition of growth. This reaction becomes particularly obvious when *flu* plants are transferred from continuous light to long day conditions (Fig.6). They stop growing but will reactivate growth once they are returned to continuous light. This response is similar to a stress

tolerance reaction shown by plants exposed to unfavorable environmental conditions. They slow down their metabolism and remain in a quiescent stage until external conditions improve again. Free tetrapyrroles are very potent photosensitizers that upon illumination may react with the ground state triplet oxygen to produce singlet oxygen (Knox and Dodge, 1985, Elstner, 1991, Arakane et al., 1996, Boo et al., 2000). Thus, the minute amounts of Pchlide that accumulate within the *flu* mutant during the dark period may be sufficient to act as a photosensitizer during reillumination. The possible release of singlet oxygen in the *flu* mutant immediately after the onset of illumination was tested *in vivo* according to Hideg et al. (1998) using dansyl-2.2.5.5-tetramethyl-2.5-dehydro-*1H*-pyrrole (DanePy) as a specific probe for singlet oxygen (Kalai et al., 1998). As shown in Fig. 7 the release of singlet oxygen occurs almost instantaneously within the first few minutes after reillumination. There are two ways of how singlet oxygen may cause a stress reaction in plants. Singlet oxygen is known to react with nucleic acids, proteins or lipids and in this way may disrupt normal cell function and trigger indirectly a broad range of pleiotropic stress responses. Alternatively it has also been proposed to act as a second messenger that activates various signaling pathways and may trigger specific stress responses (Ryter and Tyrrel, 1998). In the latter case second-site mutants of *flu* should exist that are selectively blocked in only one of the stress responses while the others are still induced as in the original *flu* mutant. Seeds of the homozygous *flu* mutant were mutagenized with EMS and screened for second-site mutants that suppress selectively only some of the stress responses induced in *flu* after a dark/light shift. Two different stress responses were used for the identification of such mutants: The rapid bleaching and death of etiolated seedlings of *flu*

during illumination and the growth inhibition of mature light-adapted plants. The analysis of more than 80 second-site mutants of *flu* revealed that singlet oxygen activates selectively several independent signal transduction pathways that can be defined genetically and that regulate stress responses and apoptosis in plants.

One major aspect of the present invention is to use these second site mutants of the *flu* mutant for the identification of key components of signal transduction pathways involved in stress responses and cell death.

A further important aspect of the present invention is that with the help of each of the identified key genes of stress-related signal transduction pathways the stress response of a plant can be manipulated in such a way that this plant is either less susceptible to this stress or is more resistant to other stressors through an activation of other stress response pathways.

A further important aspect of the present invention is that with the help of key genes involved in triggering cell death and apoptosis new targets can be defined for the development of compounds that block cell death and apoptosis. Since many genes of animals and humans are related to plant genes and can be traced back to a common origin, these targets may also facilitate the identification of compounds that display antiapoptotic activities in humans and animals.

Another aspect of the present invention is that with each of the mutants the effect of a given stress response pathway on the reaction of the whole plant can be monitored. Furthermore, the effects of blocks of this pathway at various

steps can be determined and crosslinks between this and other signal transduction pathways can be established.

Another important aspect of the present invention is that with said mutants the consequences of mutational changes in the signaling of stress responses for the overall viability and competitiveness of a given plant within a plant community can be determined, and thus targets for genetic modification can be identified that provide cultured plants with improved viability, stress tolerance and a higher productivity.

Furthermore the Flu protein itself may be used as an ideal target for a selective herbizide. Since the FLU-dependent feedback regulation of Chl biosynthesis operates only in higher plants (angiosperms) compounds that inactivate this protein should not interfere with metabolic pathways of other plants - such as gymnosperms, ferns, mosses or algae - animals, bacteria or fungi.

A further embodiment of the present invention is that the *FLU* gene in combination with the herbicide that selectively inactivates the FLU protein can also be applied as an ideal selectable marker. Mature susceptible plants that are selected against the herbicides will not die after such a treatment but instead will only transiently stop their growth and can be rescued easily after the herbicide treatment.

Another aspect of the present invention is that it offers the opportunity to construct a modified *FLU*-transgene that confers resistance of cultured plants to the herbicides of the present invention.
Many herbizides presently applied inevitably lead to the death of weeds and other species unwanted in a crop culture.

The herbizide of the present invention supports the preservation of biodiversity by putting the growth of said unwanted species "on hold" for the period of time the herbizide is applied. Once the application of the herbizide is stopped, e.g. because the crop plant reached a height sufficient not to be outgrown the susceptible plants take up their normal metabolism and grow again.

Short Description of the Figures

Figure 1 shows the relative Pchlide and Chl contents of *flu* and wt seedlings of *A. thaliana* grown in the dark (D), under nonpermissive dark to light (D → L), or permissive continuous light (LL) conditions.

Figure 2 shows a comparison of the rates of ALA synthesis (a), Pchlide (b) and heme (c) contents of wt (black bar) and *flu* (grey bar). The rates of ALA synthesis were measured in seedlings grown for 6 days in continuous light and returned to the dark for 30 min. Pchlide was measured spectroscopically and the level of free heme was measured enzymatically in etiolated seedlings. Each of the experiments was repeated three times.

Figure 3 demonstrates levels of Pchlide in etiolated seedlings of wt, *hy1*, *flu* and *hy1xflu* double mutants **(10, 12,23,31)**. Note that elevated heme levels in *hy1* antagonize the effect of the *flu* gene. FLU and heme seem to downregulate the rate of ALA synthesis independently, acting through different regulatory circuits.

Figure 4 shows Pchlide accumulation in dark-adapted mature wild-type (-) and *flu* (--) plants. Plants were grown under continuous light until they were ready to bolt. At this developmental stage plants were transferred for the first time to the dark. After 8 hr total porphyrins (a) or non-esterified porphyrins (b) were extracted. The fluorescence emission spectra of these samples were recorded using an excitation wavelength of 433 nm.

Figure 5 shows the induction of cell death in mature light-adapted *flu* plants after a dark/light shift. *flu* plants were grown initially under continuous light before they were transferred to the dark for 8 hr and reilluminated for 10 hr. Cell death was detected after trypan blue-staining of cut leaves. As controls wt plants grown under light/dark cycles and *flu* mutants grown under continuous light were tested as well.

Figure 6 shows the induction of growth inhibition of *flu* plants during a dark/light shift. All plants were initially grown under continuous light and part of these plants were kept under this light condition till their seeds could be harvested**(LL)**. The remaining plants were transferred to light/dark cycles for 29 days (L/D). wt: Control wildtype plants grown under longday conditions (L/D).

Figure 7 demonstrates the release of singlet oxygen in the *flu* mutant after a dark/light shift.
Wild-type (-) and *flu* mutants ( ) were grown under continuous light until they were ready to bolt. At this stage plants were transferred to the dark for 8 hr. Cut leaves of these plants were infiltrated with DanePy under green safe light and subsequently illuminated with white light (100 μmol

m^{-2} s^{-1}). As a further control leaves were taken from *flu* plants that were kept under continuous light without a dark treatment (--). Singlet oxygen trapping is measured as relative fluorescence quenching of DanePy (Hideg et al., 1998).

Figure 8 demonstrates the identification of the *FLU* gene. (a) Genetic and (b) physical map of the DNA region on chromosome III of *A. thaliana* that contains the *FLU* gene. (c) The region between the markers MDC16B and MLNE6 was encompassed by 17 partially overlapping cosmid clones that were used for the complementation test. Four BAC clones fully covered this region containing the *FLU* gene. (d) Details of the physical map of the nonoverlapping part of the cosmid clones 27, 28 and 48. Three open reading frames, MAG 2.6, MAG 2.7 and MAG 2.8, have been deduced by the Kazusa DNA Research Institute. (e) A schematic presentation of the structure of the FLU protein with four domains (I-IV): I, a putative chloroplast signal peptide; II, a hydrophobic region; III, a coiled coil motif; and IV, a TPR region with two TPR motives. Arrows indicate the locations of mutations in the four allelic *flu* mutants. (f) The derived amino acid sequence of FLU deduced from the *FLU* cDNA sequence. Bold letters indicate positions of mutational changes of the open reading frame that lead to the premature termination of the polypeptide chain (*) or to amino acid exchanges. Single base pair exchanges in the four allelic *flu* mutants were found by direct sequencing of PCR products amplified from genomic DNA. (g) The identification of the *FLU* gene was confirmed by the complementation of the *flu* mutant with a genomic fragment of wt DNA containing MAG 2.7.

Figure 9 shows the cDNA sequence of *FLU*.

Figure 10 shows the import of FLU into isolated chloroplasts of pea. FLU was synthesized by coupled *in vitro* transcription/translation of the *FLU* cDNA (IVT) and incubated with isolated intact chloroplasts (CP) of light-grown pea seedlings. Two major radioactively labeled protein bands of apparent MWs of 35.4 and 26.5 kDa were separated electrophoretically (-T-lysin). After thermolysin treatment of chloroplasts, only the 26.5 kDa protein was protected against proteolytic digestion and thus seemed to represent the imported mature form of the FLU protein (+ T-lysin). Following the lysis of these chloroplasts, FLU was not detectable in the stroma fraction but was bound to membranes. After extracting the membranes with an alkaline buffer (pH 11) and centrifugation at 100 000 xg for 20 min, FLU was not released to the supernatant (S) but remained tightly associated with membranes (P).

Figure 11 shows a classification of second-site mutants of *flu*. (a) etiolated seedlings of all second-site mutants accumulate similar high levels of Pchlide as the *flu* mutant. (b) growth of many of the second-site mutants is fully inhibited similar to *flu* when mature light-adapted plants are transferred from continuous light to light/dark cycles while others continue to grow under these non permissive light conditions like wt. (c) Seedlings of most of the second-site mutants (group A and C, see also Fig. 12) are no longer susceptible to photooxidative stress and accumulate similar levels of Chl when grown under non permissive light/dark cycles while *flu* and the three mutants of group B (see also Fig. 12) bleach and die.

Figure 12 shows three major groups A, B and C of second site mutants of *flu* that accumulate similar amounts of Pchlide in etiolated seedlings as *flu* (see also Fig. 1).

Figure 13 shows the characterisation of the genetically controlled suicide program of Arabidopsis seedlings.

a) The response of isolated protoplasts of *flu* (■--■) and wt (◆-◆-) to the dark/light shift. Plants were grown under continuous light before the preparation of protoplasts. Protoplasts were first incubated in the dark before they were exposed to light. The number of dead protoplasts was calculated by counting the cells that were stained by Evans'blue.

b) The response of isolated protoplasts of *flu* (■--■)and two of the second site mutants of group A ("6" ▲•••▲, "30" ◆•••◆). Isolation and incubation were done as described above under (a).

c) The response of isolated protoplasts of *flu* that were incubated with (▲--▲) or without (◆-◆)750μM hydrogen peroxide. Growth of plants and the isolation and incubation of protoplasts occurred under continuous light, such that no Pchlide accumulated.

d) The response of isolated protoplasts of *flu* and one of the second site mutants of group A to ROS released in the presence of 8μM neutral red. Protoplasts of *flu* (with ROS: ▲-▲, without ROS: Δ-Δ) and the second site mutant "6" (with ROS: ●-●, without ROS: O-O) were kept under continuous light such that no endogenous Pchlide accumulated.

Detailed description of the invention

The *FLU* gene was isolated by using a map-based cloning strategy. A detailed description of the strategies and methods that were used for the identification and isolation

of the *FLU* gene can be found in a recent publication (Meskauskiene et al. 2001). Briefly, following the fine mapping of *FLU* on chromosome 3 of Arabidopsis a contig consisting of 17 overlapping cosmid clones was generated that encompassed this chromosomal region. Each of the 17 genomic fragments was stably integrated into the genome of homozygous *flu/flu* mutants and one of them was identified that complemented the *flu* mutation. Neighbouring cosmid clones that did not complement overlapped to a large extent and left a nonoverlapping part of the positive clone that contained only a single open reading frame. The identity of this open reading frame as the *flu* gene was confirmed by first sequencing the corresponding genomic DNA of four allelic *flu*-mutants. In each case single base substitutions could be found that either lead to an amino acid exchange or created premature stop codons (Meskaukiene et al, 2001). Second a DNA fragment containing only this open reading frame complemented the *flu* mutant (Fig. 8).

A *FLU* cDNA was synthesized from cDNA derived from total RNA of *Arabidopsis* seedlings using gene specific primers. This sequence is related to the open reading frame of the clone MAG 2.7 that was released by the Kazusa-DNA-Research Institute and that was labeled as "similar to unknown protein". However, there are several minor differences between the cDNA shown in Fig.9 and the published genomic DNA. The ORF of the *FLU* cDNA predicts a protein of 316 amino acids that is unrelated to any of the enzymes known to be involved in tetrapyrrole biosynthesis and that has not been previously described. Nevertheless, the predicted FLU protein reveals features that may be important for its presumptive function during feedback control. It contains an N-terminal extension that resembles import signal sequences of nuclear-

encoded plastid proteins (Fig. 8 e, f). Furthermore, the central part of FLU ranging from amino acid position 125 to 146 consists of a hydrophobic domain, which may be important for anchoring the protein within a membrane, while the C-terminal part is hydrophilic (Fig. 8 e, f). We tested these predictions deduced from the sequence of the *FLU* gene. FLU was synthesized in a coupled transcription/translation system in the presence of [^{35}S]-methionine and then imported into chloroplasts isolated from 12-day-old pea seedlings. At the end of the incubation the plastid proteins were dissolved and separated electrophoretically. Two major radioactively labeled proteins could be detected by autoradiography, one with an apparent molecular weight of 35.4 kDa similar to the size of the *in vitro* synthesized product of the *FLU* gene and the second with an apparent MW of 26.5 kDa (Fig. 10). After treatment of chloroplasts with thermolysin most of the 35.4 kDa polypeptide had been digested, while the 26.5 kDa protein was protected against proteolytic attack. This latter protein seems to represent the imported and processed form of the FLU protein (Fig. 10). After import, chloroplasts were lysed and the membranes separated from the soluble stroma fraction by centrifugation. FLU was recovered only in the membrane but not in the stroma fraction (Fig. 10). When the membranes were extracted with an alkaline buffer (200 mM Na_2CO_3, pH 11), the FLU protein was not released but remained tightly associated with plastid membranes (Fig. 10).

Data base searches indicate that the hydrophilic half of FLU contains two different regions implicated in protein-protein interactions. Two tetratricopeptide repeats (TPRs) were predicted for the C-terminal region. This interpretation fits with the observed amino acid substitution in the *flu* 1-1 mutant. The alanine residue at position 20 in the first

predicted TPR motif is one of the few conserved amino acids of TPR motives (Das et al., 1998). Its replacement by a valine leads to the inactivation of FLU. The second region possibly engaged in protein-protein interaction is a short coiled coil motif adjacent to the hydrophobic membrane anchor of FLU. Notably, the amino acid substitution in *flu* 1-4 is found in this region.

Besides heme, also Pchlide has been proposed to act as a regulator of tetrapyrrole biosynthesis and to inhibit one of the steps leading to ALA formation. Pchlide has been shown to be localized in the hydrophobic environment of prolamellar bodies, plastid envelopes and thylakoid membranes, whereas the target enzymes of feedback control, Glu tRNA reductase and Glu 1-semialdehyde aminotransferase (GSA), are found in the stroma. Direct interaction between Pchlide and these enzymes may not be feasible. The FLU protein could be necessary to bridge the gap between the membranes and the stroma and to facilitate the interaction between the putative effector of feedback inhibition and hydrophilic target enzymes. Within the membrane Pchlide may associate with the hydrophobic membrane anchor of FLU, whereas the hydrophilic part of FLU with its two putative protein-interacting domains may interact with the stroma enzymes.

The extent of damage caused by the *flu*-mutation very much depends on the developmental stage of the mutant plant. In seedlings that have been transferred from the dark to the light the accumulation of free Pchlide as a result of FLU inactivation leads to a strong photooxidative damage that is lethal to the plant. However, in more mature light-adapted green plants the effect of FLU inactivation is less severe.

Following a brief dark incubation of 6-8, preferably 8 hours there is no immediate photooxidative damage visible and the Chl content of such plants does not change. Excitation of minor amounts of free Pchlide that accumulate in these plants during the dark period leads to the rapid transient release of singlet oxygen. This reactive oxygen species activates signal transduction pathways that induce various stress responses. The most obvious stress responses in the mature plants are the spontaneous formation of necrotic lesions on leaves and rapid inhibition of growth.

An important aspect of the present invention is that it allows the identification of genes that form part of signal transduction pathways involved in triggering stress responses and apoptosis. Several of these genes may serve as targets for a genetic improvement of stress resistance in cultured plants while others can be used to delay or prevent the induction of cell death. Since many genes in plants and animals are remarkably similar in their structure and function antiapoptotic genes of Arabidopsis may potentially also be of interest for the identification of similar genes in animals and humans.

A genetic approach has been used to identify such genes. *flu* mutants were mutagenized a second time and revertants were identified that were still able to overaccumulate Pchlide in the dark but did not show the stress responses of the original *flu* mutant. Two major stress response traits were used for the screening of the second-site mutants: The rapid death of etiolated seedlings of *flu* after the transfer from the dark to the light and the inhibition of growth of mature plants.

At the seedling stage revertants of *flu* were identified that green normally after a dark/light shift even though etiolated seedlings accumulate similar amounts of free Pchlide as the *flu* mutant and thus release singlet oxygen during illumination (Fig. 11). At a more mature stage some of these mutants react like *flu* and are fully inhibited during illumination while others continue to grow normally like wildtype plants (Fig. 10). The second group of revertants was identified at the rosette leaf stage shortly before the plants start to bolt. In contrast to the *flu* mutants that stop to grow under light/dark conditions these second-site mutants continue to grow like wildtype plants, even though singlet oxygen is still being released. Some of these revertants are fully susceptible to light stress at the seedling stage and die rapidly after transfer from the dark to the light similar to the original *flu* mutant (Fig. 11) while others green normally at the seedling stage, even though they have been kept under nonpermissive dark/light conditions (Fig. 11). Collectively these results demonstrate that singlet oxygen activates several independent signal transduction pathways that can be defined genetically and that regulate stress responses and cell death of plants. Second-site mutants of *flu* can be exploited for the identification of key components of these signal transduction pathways.

This invention is further illustrated by the following examples.

Example 1

As a first step towards its functional characterization, a map-based strategy to isolate *FLU* has been used. The

inventors genetically mapped *FLU* in F2 plants from a cross between the *flu/flu* mutant derived from Arabidopsis ecotype *Landsberg erecta* and wildtype *FLU/FLU* plants of ecotype *Columbia*. Tests on 80 plants for genetic linkage between the *flu* phenotype and cleaved amplified polymorphic sequence (CAPS) markers placed *FLU* on chromosome 3 flanked by the markers g 4711 and GAPC (Fig. 8a). Since this chromosomal position is different from that of the *HEM A 1* and *HEM A 2* genes that encode the Glu tRNA reductase isozymes of Arabidopsis (Kumar and Söll, 2000), the *FLU* gene does not code for this putative target enzyme that has been proposed previously to interact directly with a tetrapyrrole intermediate (Pontoppidan and Kannangara, 1995).

For the subsequent fine mapping the size of the mapping population was increased to 960 F 2 plants. *FLU* was located on a genomic fragment of approximately 210 kb flanked by the polymorphic markers MDC16B and MLNE6 (Fig. 8 b). Four overlapping bacterial artificial chromosome (BAC) clones, F5L21, F21019, F25F10 and F25F9, encompassed this fragment. The four BAC clones were partially digested with Hind III and the resulting DNA fragments of an average size of 15 to 25 kb were cloned in the binary cosmid vector pBIC 20 (Meyer et al., 1994). From this library a contig consisting of 17 overlapping cosmid clones was generated that encompassed the 210 kb chromosomal region including the *FLU* gene (Fig. 8c). Each of the 17 genomic fragments was stably integrated into the genome of homozygous *flu/flu* plants using *Agrobacterium tumefaciens* for transformation (Bechtold et al., 1993). Integration of the complete genomic fragments was verified by the expression of the kanamycin resistance gene (NPT II) and the GUS reporter gene that were positioned on the vector DNA next to the right and the left border of the genomic DNA

insert, respectively. Seeds from each of the primary transformants were collected and germinated under light/dark cycles. The *flu* phenotype could easily be scored by the photobleaching of the homozygous mutant plants (Fig. 1). One of the cloned genomic fragments (clone 28) complemented the *flu* mutant. Among eight transformants that contained this fragment two segregated the wildtype phenotype in a 3 : 1 ratio, while the other plants showed segregation ratios between 5 :1 to 30 : 1, indicating that more than one copy of the transgene was present in these plants. The neighboring cosmid clones 27 and 48 of the contig that overlapped to a large extent with the insert of the cosmid clone 28 did not complement the *flu* mutant. The nonoverlapping part of the complementing genomic fragment has a size of 1.4 kb. It contains a single open reading frame (MAG 2.7) (Fig. 8d) (http://www.kazusa.or.jp/). Specific primers for this and the two adjacent genes (MAG 2.6, MAG 2.8) were used to generate gene-specific probes for the detection of the corresponding transcripts in the four allelic *flu* mutants by Northern blot analysis. Two of the three genes, MAG 2.6 and 2.7, were expressed. While the transcript levels of the MAG 2.6 gene were similar in all four mutants and the wildtype, transcripts of the MAG 2.7 gene were detectable only in the *flu* 1-1 and *flu* 1-4 mutants and in wildtype plants. In the *flu* 1-2 and 1-3 mutants, however, transcripts of this gene were not detectable. The DNA regions of all four allelic *flu* mutants covering the open reading frame of this gene and an additional 200 bp upstream region were sequenced. In all four mutants single point mutations were detected in this region. In two of the mutants, *flu* 1-2 and 1-3, these changes led to the formation of two new stop codons within the open reading frame, while in the mutants *flu* 1-1 and 1-4 single base

exchanges resulted in an amino acid exchange from Ala to Val (Fig. 8e,f).

A 4.6 kb genomic fragment of wt DNA containing the ORF of MAG 2.7, a 1.7 kb promoter region, and a 1.5 kb downstream sequence, was used for a final complementation test to confirm the identification of the *FLU* gene. Primary transformants were selected on kanamycin. Seeds of these plants were collected and germinated under light/dark cycles. Seedlings of nontransformed *flu* plants were photobleached under these conditions, whereas seedlings of *flu* complemented with MAG 2.7 segregated the wt phenotype in a 3 : 1 ratio (Fig. 8 g).

Example 2

Following the screening strategy described above 65 second-site mutants have been selected, all of which accumulate similar high amounts of Pchlide in the dark as the etiolated *flu* seedlings and thus upon illumination release similar amounts of singlet oxygen. Three groups of mutants have been distinguished (Fig. 12). The first group A is no longer susceptible to photooxidative stress at the seedlings stage whereas bolting of the plant is fully inhibited at the rosette leaf stage (Fig. 12). Mutants of this group define a genetic program that initiates cell death after the release of singlet oxygen. The mutants have lost the capacity to perceive death signals and to initiate apoptosis. The second group B contains mutants with an inverse phenotype. Growth at the rosette leaf stage is no longer inhibited whereas the seedlings rapidly die when transferred from the dark to the light. Mutants of this group define genetically a signaling pathway that activates a stress tolerance program. Mutants of this group apparently have lost the ability to sense stress

signals and do not slow down their growth. Members of the third group C grow normally at the rosette leaf stage and upon illumination etiolated seedlings are no longer damaged and green similar to wildtype. Mutants of this group define genes that act close to the initial release of singlet oxygen. The mutated genes of members of group B and C offer an attractive possibility to increase the apparent resistance of plants against stressors. Stress reactions appear not to be only the result of injuries inflicted upon plants through the physical environmental conditions of plants. Instead stress reactions seem to be regulated also by a genetically controlled program that anticipates a worsening of environmental conditions and improves the plant's ability to withstand such stresses. Mutants of group B and C apparently have lost the ability to interpret environmental cues and continue to grow normally. Under natural conditions such lack of sensing would be detrimental to the plant because it would not be prepared for a worsening of its environmental conditions. In the case of cultured plants, however, such genetic modification may eventually increase the plant's yield by making it less sensitive to environmental stress and allows it to relocate its resources into seeds before these will be harvested.

Example 3

The mutated *flu* gene and the mutant plant were used for the characterization of stress and cell death reactions induced by reactive oxygen. After switching the plant from darkness to light a quick pulse of singulet oxygen is set free, which induces various stress and cell death reactions. These reactions are visible either in the plant (e.g. growth

inhibition of light adapted plants, necrosis, or death of seedlings) or may be seen at the level of gene expression.

After a second mutagenesis of the *flu* mutant, second site mutants were identified, which either did not show any of the above cited stress reactions (growth inhibition, stress, death of seedlings) or did show only one or two of these stress reactions. Since in all of the isolated second site mutants singlet oxygen was set free after a dark/light treatment, it was concluded that most of the stress reactions are genetically controlled and cannot be explained as being caused by unspecific cell or tissue injuries induced by reactive oxygen.

With the help of the second site mutants it was possible to define wildtype genes, which participate in the genetic control of stress reactions. The importance of these genes for stress reactions induced by biotic or abiotic stressors was determined as follows:

a) growth of second site mutants on soil under continuous light until rosette leaf stage;
b) exposing said mutants to abiotic and biotic stressors while illuminated;
c) identification of second site mutants showing no symptoms of stress;
d) assigning said second site mutants to specific stress induced signal transduction pathways.

The various stressors being tested were biotic stressors such as bacterial, fungal and viral pathogens as well as abiotic stressors such as light, heat, cold, salt, dryness and wound stress.

Example 4

The above described second site mutants were also applied for the identification of genes playing key roles in the stress related signal signal transduction network.

a) *Flu* mutants were transferred to darkness after growth under continuous light conditions for a short period of time (6-10 hours, preferably 8 hours). After this period of darkness the *flu* mutants were exposed to light for 0, 10, 20, 60 minutes and 2, 4, 8, 12, 24 hours. RNA samples were isolated and compared on Affymetrix-Arabidopsis DNA chips with wildtype control RNA regarding expression differences induced by singlet oxygen set free in the *flu* mutants. Early inducible genes (maximum of gene expression within 20 to 30 minutes) and late inducible genes (maximum between 4 and 12 hours) were identified.

b) All of the under a) identified genes inducible by singlet oxygen were combined on a microarray plate. With these plates changes of stress gene activity in second site mutants were determined, which have been treated with biotic and abiotic stressors. The second site mutants were grown under continuous light until the rosette leaf stage and were still kept under continuous light during exposure to the various stressors. After various periods of time depending on the respective stressor, RNA samples were prepared. These samples were labeled with fluoreszence colours and hybridized with RNA samples from *flu* mutants treated as described for the second site mutants. Since the control RNA was labeled with a different fluoreszence colour, differences in gene stimulation between second site mutant RNA and control *flu*

mutant RNA could be determined from the relation of the two fluoreszent colours.

It could be shown that in many of the second site mutants after a treatment with a natural stressor the induction of a gene is comparable with that of the *flu* mutant. However, in contrast to *flu* in various second-site-mutants expression of a given gene has not been induced, whereas in other second-site-mutants the same gene was induced to a much higher extent than in the *flu* control.

By superimposing numerous of such "fingerprints" the effect of genes defined by the second-site-mutations on changes in the expression of single stress response genes after treatment with biotic and abiotic stressors was determined. In this way second-site-mutations could be identified which interact with various signal transduction pathways and which represent genetic "coordination points".

The genes identified as described above may serve as preferred targets for the manipulation of signal transduction caused by various stresses.

Example 5

The above described strategies for the identification of key genes in the signal transduction of various stresses was slightly altered and applied in order to identify genes controlling cell death and apoptosis:

Seedlings of *flu* mutants and second site mutants were incubated on agar plates in continuous light for four days and afterwards transferred to darkness for 8 hours. After

reexposing them to light, seedlings of the *flu* mutant showed extensive necrosis and bleaching, whereas the selected second site mutants grew as normal as the wildtype seedlings eventhough they accumulated high amounts of Pchlid like the *flu* mutant and activated various stress genes, which were expressed in the *flu* mutant as well but not in the wildtype seedlings. In addition, many of the selected second site mutants showed in the rosette leaf stage growth inhibtion just like the *flu* mutant. Therefore, these second site mutants define genes, which, regarding their wildtype allels, are part of a genetic „suicide program" inducing cell death after exposure to oxidative stress.

The same experiments were carried out with protoplasts of wildtype, *flu* mutant and second site mutants as described in example 6.

Example 6

The mutations of group A (compare Example 2) define a genetically controlled suicide program that seems to be activated following the release of singlet oxygen (Fig. 12). The genes that form part of this program were tested in the following way. First, protoplasts were isolated from *flu* plants grown under continuous light. These protoplasts were then kept in the dark for 6-8, preferably 8 hours to allow the accumulation of Pchlide before they were exposed to light. During the next 7 hours of illumination these protoplasts died as indicated by Evans blue staining and microscopic inspection (Fig. 13a). Protoplasts isolated from control wildtype plants that were subjected to the same dark/light treatment did not show such a loss in viability

during reillumination. Similar experiments were repeated with protoplasts isolated from two of the revertants that belong to group A. In contrast to protoplasts of the *flu* mutant, these protoplasts did not show any drastic loss in viability during illumination, even though they had accumulated Pchlide during the preceding dark period (Fig 13b). In the final experiments the antiapoptotic activity of these mutated genes of group A was tested without the interference of the *flu* gene. First, protoplasts of *flu* plants grown under continuous light were isolated and incubated under continuous illumination without any intervening dark period, such that no Pchlide could accumulate within these cells. These protoplasts maintained their full viability during the incubation (13c). In parallel assays these protoplasts were incubated with hydrogen peroxide. They rapidly died in the presence of ROS (Fig. 13c). However, when protoplasts of mutants of group A were tested they maintained their viability in the presence of ROS much more than *flu* protoplasts (Fig. 13d). Two important conclusions can be drawn from these experiments. First, the wildtype allels of genes defined by the group A mutants are normally required for triggering cell death responses to oxidative stress. Second, inactivation of these genes by mutations block one or several signaling pathways that lead to cell death. Thus, genes and their products defined by group A mutations can be exploited as a potential target for compounds that mediate antiapoptotic activities.

Since there is a high homology between numerous plant and mammalian genes, the results and applications of the present invention are transferable to the mammalian system.

References

Alscher, R. G., Donahue, J. L., and Cramer, C. L. (1997). Reactive oxygen species and antioxidants: Relationships in green cells. Physiol. Plantarum **100**, 224-233.

Apostol, I., Heinstein, P. F., and Low, P. S. (1989). Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol. **90**, 109-116.

Arakane, K., Ryu, A., Hayashi, C., Masunaga, T., Shinmoto, K., Mashiko, S., Nagano, T., and Hirobe, M. (1996). Singlet oxygen ($^1\Delta_g$) generation from coproporphyrin in *Propionibacterium acnes* on irradiation. Biochem. Biophys. Res. Commun. **223**, 578-582.

Beale, S. I., and Weinstein, J. D. (1990) Tetrapyrrole metabolism in photosynthetic organisms. In: Dailey HA (ed) Biosynthesis of Heme and Chlorophyll, pp 287–391. McGraw-Hill, New York.

Bechtold, N., Ellis, J. and Pelletier, G. (1993). In planta Agrobacterium mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C.R. Acad. Sci. Paris, Life Sciences, 316, 1194-1199.

Boo, J. C., Lee, K. P., and Jung, J. (2000). Rice plants with a high protochlorophyllide accumulation show oxidative

stress in low light that mimics water stress. J. Plant Physiol. **157**, 405-411.

Bowler, C., and Fluhr, R. (2000). The role of calcium and activated oxygens as signals for controlling cross tolerance. Trends Plant Sci. **5**, 241-245.

Chakraborty, N., and Tripathy, B. C. (1992). Involvement of singlet oxygen in 5-aminolevulinic acid-induced photodynamic damage of cucumber (*Cucumis sativus* L.) chloroplasts. Plant Physiol. **98**, 7-11.

Dangl, J. L., Dietrich, R. A., and Richberg, M. H. (1996). Death don't have no mercy: Cell death programs in plant-microbe interactions. Plant Cell **8**, 1793-1807.

Das, A. K., Cohen, P. T. W., and Barford, D. (1998) The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. EMBO J. 17, 1192-1199.

Duggan, J., and Gassman, M. L. (1974) Induction of porphyrin synthesis in etiolated bean leaves by chelators of iron. Plant Physiol 53: 206–215.

Elstner, E. F. (1991). Mechanisms of oxygen activation in different compartments of plant cells. In Active oxygen/oxidative stress in plant metabolism. E.J. Pelland and K.L. Steffen, eds. (Rockville, MD: American Society of Plant Physiologists) pp. 13-25.

Fluhr, R., Harel, E., Klein, S., and Meller, E. (1975) Control of aminolevulinic acid and chlorophyll accumulation

in greening maize leaves upon light-dark transitions. Plant Physiol 56: 497–501.

Foyer, C. H., and Harbinson, J. C. (1994). Oxygen metabolism and the regulation of photosynthetic electron transport. In Causes of photooxidative stress and amelioration of defense systems in plants, C.H. Foyer and P.M. Mullineaux, eds. (Boca Raton, Fl.: CRC Press) pp. 1-42.

Granick, S. (1959) Magnesium porphyrin is formed in barley seedlings treated with delta-aminolevulinic acid. Plant Physiol Suppl 34: 18.

Hansson, M., Gough, S. P., Kannangara, C. G., and v. Wettstein, D. (1997) Analysis of RNA and enzymes of potential importance for regulation of 5-aminolevulinic acid synthesis in the protochlorophyllide accumulating barley mutant *tigrina-d*^{12}. Plant Physiol. Biochem. 35, 827-836.

Hideg, E., Kalai, T., Hideg, K., and Vass, I. (1998). Photoinhibition of photosynthesis in vivo results in singlet oxygen production. Detection via nitroxide-induced fluorescence quenching in broad bean leaves. Biochemistry **37**, 11405-11411.

Hu, G., Yalpani, N., Briggs, S. P., and Johal, G. S. (1998). A porphyrin pathway impairment is responsible for the phenotype of a dominant disease lesion mimic mutant of maize. Plant Cell **10**, 1095-1105.

Ilag, L. L., Kumar, A. M., & Söll, D. (1994) Light regulation of chlorophyll biosynthesis at the level of 5-

aminolevulinate formation in Arabidopsis. Plant Cell 6: 265-275.

Kalai, T., Hideg, E., Vass, I., and Hideg, K. (1998). Double (fluorescent and spin) sensors for detection of reactive oxygen species in the thylakoid membrane. Free Rad. Biol. Med. **24**, 649-652.

Karpinski, S., Escobar, C., Karpinska, B., Creissen, G., and Mullineaux, P. M. (1997). Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell **9**, 627-640.

Karpinski, S., Reynolds, H., Karpinska, B., Wingsle, G., Creissen, G., and Mullineaux, P. M. (1999). Systemic signaling and acclimation in response to excess excitation energy in *Arabidopsis*. Science **284**, 654-657.

Knox, J. P., and Dodge, A. D. (1985). Singlet oxygen and plants. Phytochemistry **24**, 889-896.

Kovtun, Y., Chiu, W.-L., Tena, G., and Sheen, J. (2000). Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc. Natl. Acad. Sci. USA **97**, 2940-2945.

Kumar, A.U. and Söll, D. (2000). Antisense *HEMA 1* RNA expression inhibits heme and chlorophyll biosynthesis in Arabidopsis. Plant Physiol. 122, 49-55.

Levine, A.M., Tenhaken, R., Dixon, R.A., and Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant

hypersensitive disease resistance response. Cell **79**, 583-593.

Low, P. S., and Merida, J. R. (1996). The oxidative burst in plant defense: Function and signal transduction. Physiol. Plantarum **96**, 533-542.

Mach, J. M., Castillo, A. R., Hoogstraten, R., and Greenberg, J. T. (2001). The *Arabidopsis*-accelerated cell death gene *ACD2* encodes red chlorophyll catabolite reductase and suppresses the spread of disease symptoms. Proc. Natl. Acad. Sci. USA **98**, 771-776.

Malan, C., Gregling, M. M., and Gressel, J. (1990). Correlation between CuZn superoxide dismutase and glutathione reductase and environmental and xenobiotic stress tolerance in maize inbreds. Plant Sci. **69**, 157-166.

Matringe, M., Camadro, J.M., Labbe, P., and Scalla, R. (1989). Protoporphyrinogen oxidase as a molecular target for diphenylether herbicides. Biochem. J. **260**, 231-235.

Meyer, K., Leube, M. and Geill, E.,(1994). A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. Science 264, 1452-1455, 1994)

Meskauskiene, R., Nater, M., Goslings, D., Kessler, F., op den Camp, R. and Apel, K. (2001) Flu a negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. PNAS, 98, 12826-12831.

Mock, H.-P., Keetman, U., Kruse, E., Rank, B., and Grimm, B. (1998). Defense responses to tetrapyrrole-induced oxidative

stress in transgenic plants with reduced uroporphyrinogen decarboxylase or coproporphyrinogen oxidase activity. Plant Physiol. **116**, 107-116.

Mock, H.-P., Heller, W., Molina, A., Neubohn, B., Sandermann, H., and Grimm, B. (1999). Expression of uroporphyrinogen decarboxylase or coproporphyrinogen oxidase antisense RNA in tobacco induces pathogen defense responses conferring increased resistance to tobacco mosaic virus. J. Biol. Chem. **274**, 4231-4238.

Molina, A., Volrath, S., Guyer, D., Maleck, K., Ryals, J., and Ward, E. (1999). Inhibition of protoporphyrinogen oxidase expression in *Arabidopsis* causes a lesion-mimic phenotype that induces systemic acquired resistance. Plant J. **17**, 667-678.

Nadler, K., and Granick, S. (1970) Controls on chlorophyll synthesis in barley. Plant Physiol. 46, 240-246.

Nielsen, O. F. (1974) Macromolecular physiology of plastids. XII. *Tigrina* mutants of barley. Hereditas 76: 269–304.

Orozco-Cardenas, M., and Ryan, C. A. (1999). Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proc. Natl. Acad. Sci. USA **96**, 6553-6557.

Pontoppidan, B. and Kannangara, C.G. (1995). Purification and partial characterization of barley glutamyl-tRNA ^{Glu}reductase, the enzyme that directs glutamate to chlorophyll biosynthesis. Eur. J. Biochem. 225, 529-537.

Prasad, T. K., Anderson, M. D., Martin, B. A., and Stewart, C. R. (1994). Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell **6**, 65-74.

Rebeiz, C. A., Montazer-Zouhoor, A., Mayasich, J. M., Tripathy, B. C., Wu, S.-M., and Rebeiz, C. (1988). Photodynamic herbicides. Recent developments and molecular basis of selectivity. CRC Critical Rev. Plant Sci. **6**, 385-436.

Ryter, S. W. and Tyrrell, R.-M. (1998). Singlet molecular oxygen($^1O_2$): A possible effector of eukaryotic gene expression. Free Rad. Biol. Med. **24**, 1520-1534.

Tsugane, K., Kobayashi, K., Niwa, Y., Ohba, Y., Wada, K., and Kobayashi, H. (1999). A recessive Arabidopsis mutant that grows photoautotropically under salt stress shows enhanced active oxygen detoxification. Plant Cell **11**, 1195-1206.

v. Wettstein. D., Gough, S., and Kannangara, C. G. (1995)) Chlorophyll biosynthesis. Plant Cell 7: 1039—1057.

Wu, G., Shortt, B. J., Lawrence, E. B., Levine, E. B., Fitzsimmons, K. C., and Shah, D.M. (1995). Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell **7**, 1357-1368.

CLAIMS

1. A gene in angiosperms, characterized in that said gene is the *Flu* gene of Arabidopsis thaliana and/or any other homologue gene in angiosperms.

2. The gene according to claim 1, characterized in that said gene is coding for a protein regulating the tetrapyrrole biosynthesis in angiosperms.

3. A mutant gene in angiosperms, characterized in that said mutant gene is a *Flu* mutant gene and/or any other homologue gene in Arabidopsis thaliana.

4. The mutant gene of claim 3, characterized in that the mutation causes photosensitivity of plants carrying said mutant gene after switching from a period of darkness to a period of light.

5. The mutant gene according to claim 4, selected from the group consisting of *flu* 1-1, *flu* 1-2, *flu* 1-3 and *flu* 1-4.

6. The mutant *Flu* gene according to claim 5, wherein said gene is applied in the identification of stress and/or apoptosis reactions induced by singlet oxygen.

7. The mutant *Flu* gene according to claim 6, wherein the stress and/or apoptosis reactions are identified in plants comprising said mutant gene by growth inhibition, necrosis and/or death of seedlings.

8. The mutant *Flu* gene according to claim 6, wherein the stress and/or apoptosis reactions are identified by gene expression.

9. A second site *Flu* mutant gene, characterized in that plants comprising said second site mutant do not or only partly show stress and/or apoptosis reactions like growth inhibition, necrosis and/or death of seedlings.

10. The second site mutant according to claim 9, applied for the identification of wildtype alleles genetically controlling stress reactions.

11. A method for identifying non-mutated wildtype alleles of claim 10 genetically controlling stress reactions comprising the following steps:

e) growth of second site mutants on soil under continuous light until rosette leaf stage;

f) exposing said mutants to abiotic and biotic stressors while illuminated;

g) identification of second site mutants showing no symptoms of stress;

h) assigning said second site mutants to specific stress induced signal transduction pathways.

12. The method according to claim 11, wherein the biotic stressors are selected from the group consisting of viral, bacterial and fungal pathogens.

13. The method according to claim 11, wherein the biotic stressors are selected from the group consisting of salt, heat, cold, light, drought and wound stress.

14. The second site mutant gene according to claim 9, applied as a target for the development of active compounds specifically decreasing or inhibiting stress reactions in plants.

15. The second site mutant gene according to claim 9, applied as a tool for the identification of stress induced signal transduction networks.

16. A method for the identification of stress induced signal transduction pathways, comprising the following steps:

a) transferring *flu* mutant plants grown in continuous light for a period of 6 to 10 hours to darkness;
b) exposing the *flu* mutant plants to light periods of 0, 10, 20, 60 minutes, 2, 4, 8, 12 and 24 hours;
c) isolating RNA samples;
d) comparing the *flu* mutant RNA with controls of wildtype RNA on Arabidopsis DNA chips; and
e) evaluating early and late expression of the genes induced by singlet oxygen;
f) combining all genes inducible by photostress identified in step e) on one microarray plate;
g) determining changes of stress gene activity using the plates of step f) in RNA of second site mutant plants, which have been treated with biotic and abiotic stressors while growing under continuous light.

17. A method for the identification of genes involved in the control of cell death and apoptosis, comprising the steps of:

a) incubating *flu* mutant and second site mutant seedlings 4 to 6 days in continuous light;

b) transferring the seedlings of step a) into darkness for a period of 6 to 10 hours and exposing them to light;

c) identifying second site mutant seedlings with normal appearance that accumulate high levels of Pchlide in the dark, but in contrast to *flu* do not bleach and die during illumination.

18. The method of claim 17, comprising the steps of:

a) incubating protoplasts of wildtype, *flu* mutants and second site *flu* mutants under continuous light;

b) incubating the protoplasts of step a) with low concentrations of compounds that cause oxidative stress such as e.g. neutral red or H_2O_2.

c) distinguishing wildtype and *flu* mutant protoplasts dying from this treatment from second site *flu* mutant protoplasts with normal appearance.

19. The method of claim 18, applied for the identification of genes responsible for cell death reactions.

20. A method for the identification of compounds inactivating or blocking genes responsible for cell death reactions in plants and mammalians.

21. The *Flu* gene and its homologues according to claim 1 used as a target for herbizides specific for angiosperms.

22. A herbizide specific for the *Flu* gene and its homologues.

23. Use of the *Flu* gene and its homologues of claim 1 in combination with the herbicide of claim 23 as a selectable marker.

24. A modified *Flu* gene conferring resistance to the herbicide according to claim 22.

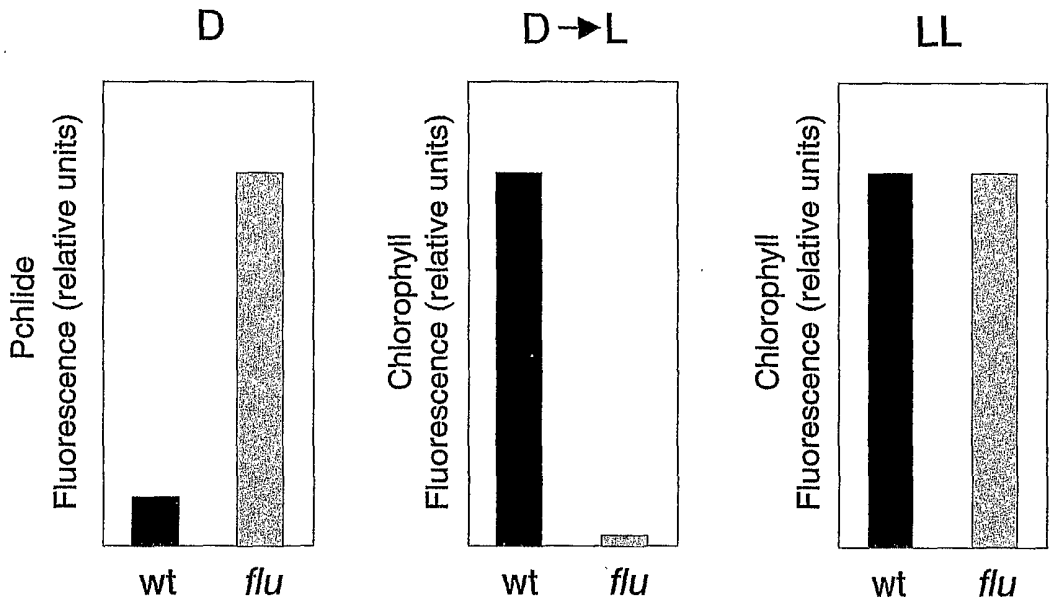
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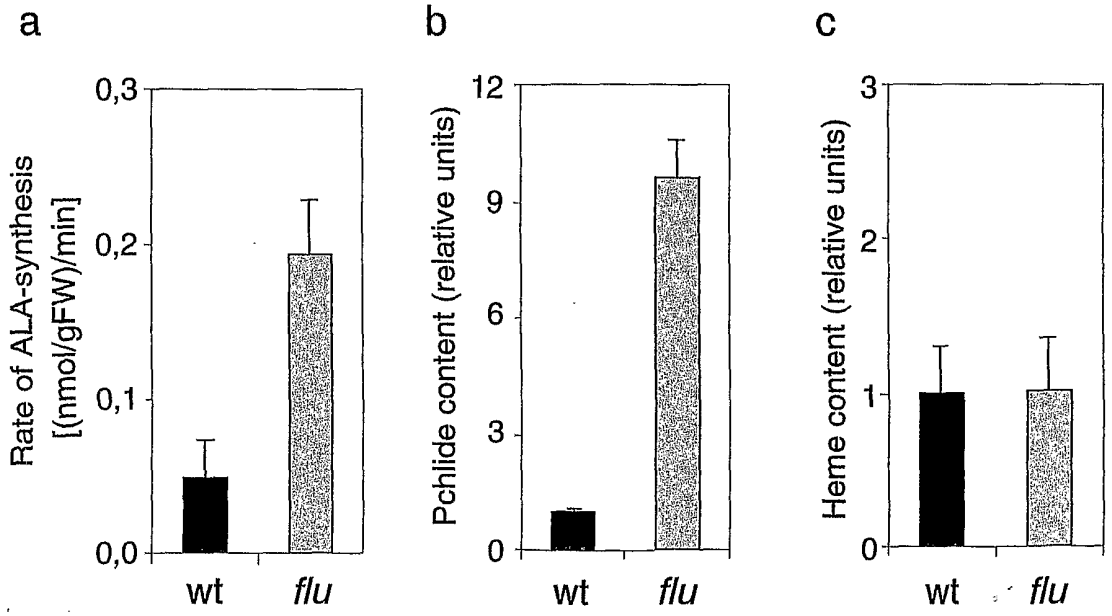
Fig.1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Pchlide content (relative units)
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9
6
3
0
Heme content (relative units)
3
2
1
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wt
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flu

Fig.2

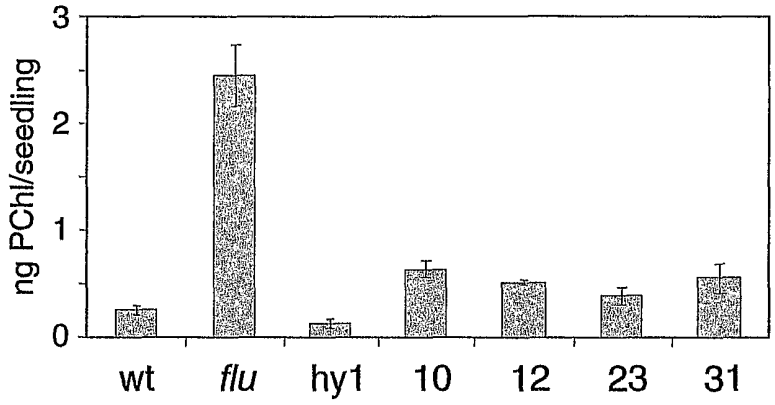

ng PChl/seedling
3
2
1
0
wt
flu
hy1
10
12
23
31

Fig. 3

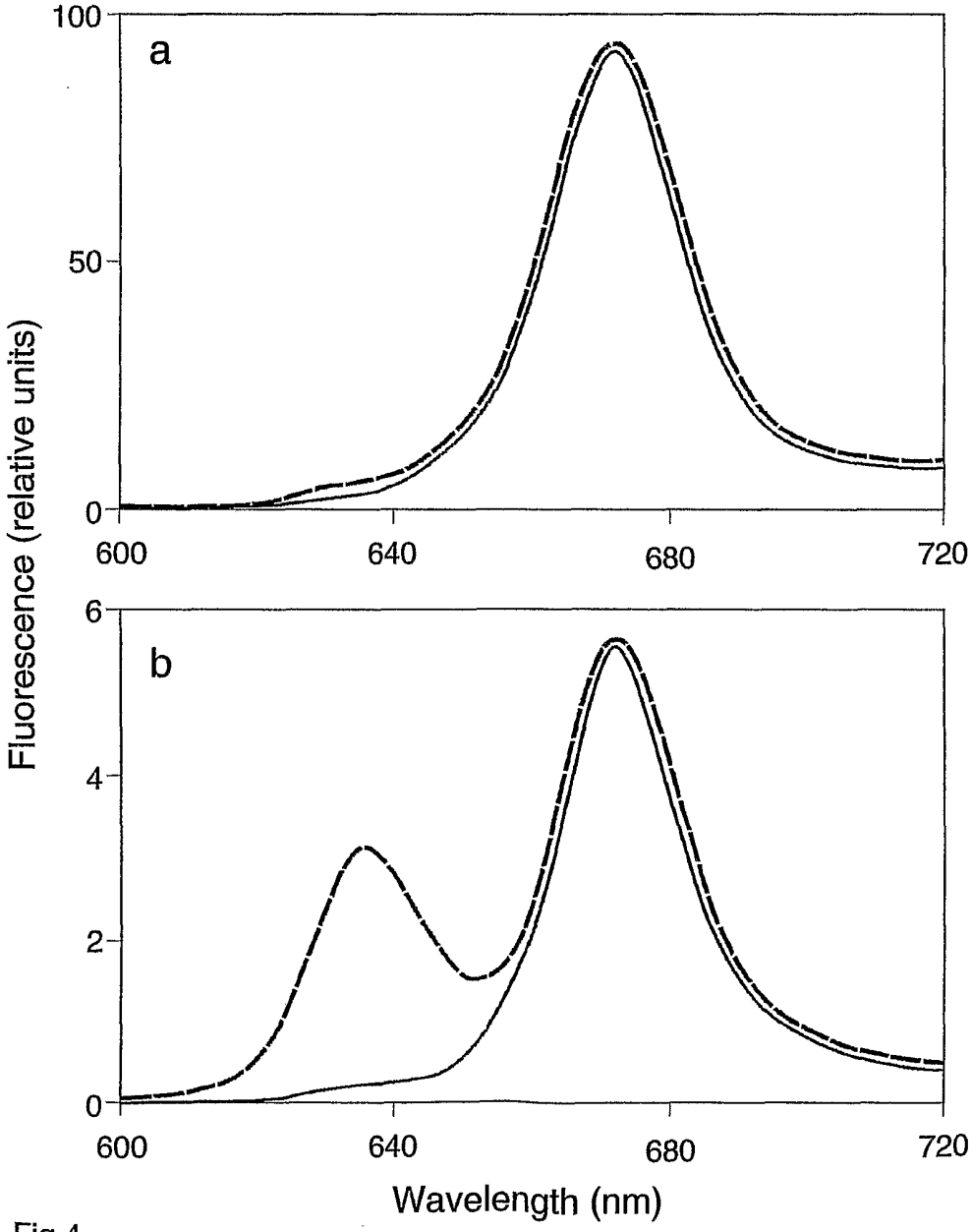

a
b
Fluorescence (relative units)
100
50
0
6
4
2
0
600
640
680
720
Wavelength (nm)

Fig.4

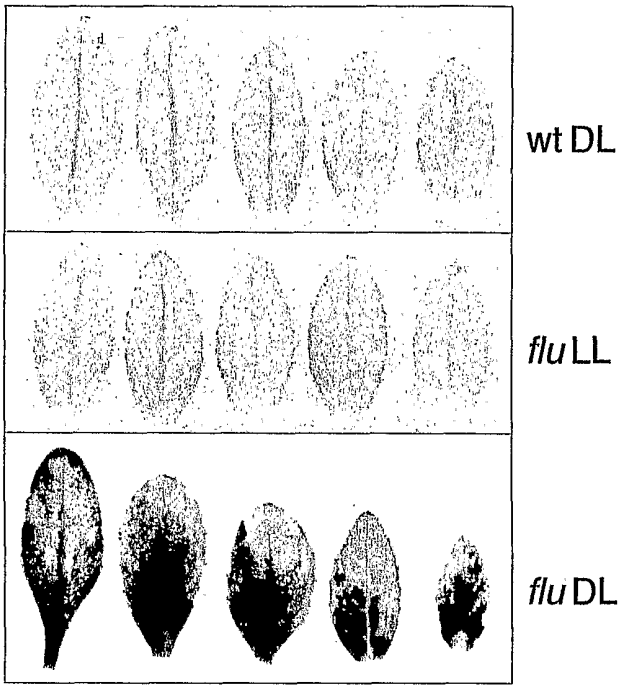


Fig.5

Fig.6

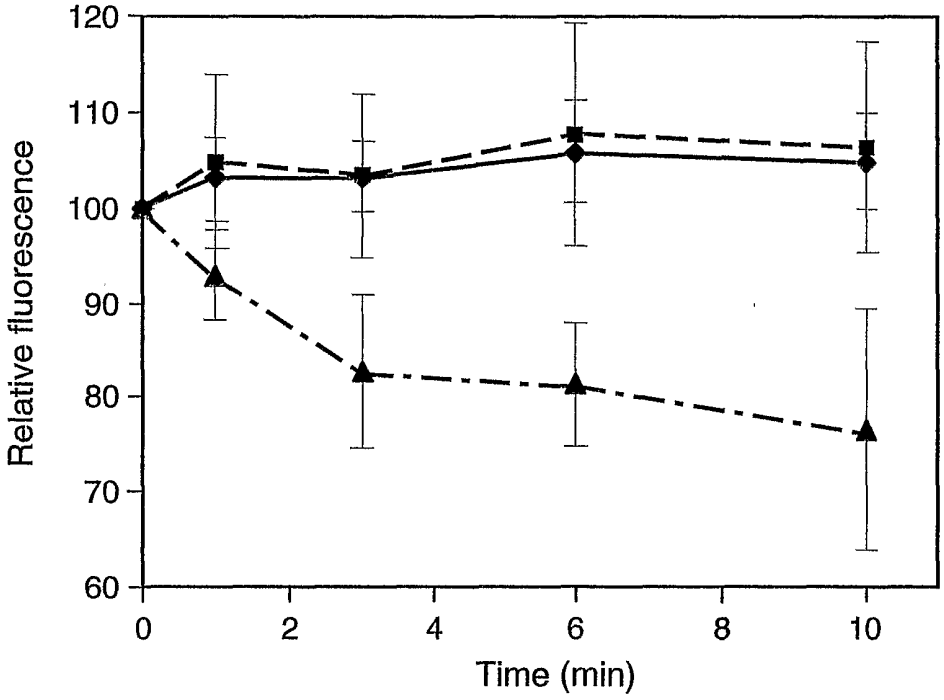

120
110
100
90
80
70
60
Relative fluorescence
0
2
4
6
8
10
Time (min)

Fig. 7

Chromosome III

a

GAPC ATHCHIB mi289 g4711

FLU

1cM

b

FLU

MDC16B nga162 MLNE6

No. of recombination events 18 3 0 0 0 3 6 7

100kb

c

27

28

48

10 kb

d

27 28 48

MAG2.6 MAG2.7 MAG2.8

e

N - terminus C - terminus

I II III IV IV

*flu*1-3 *flu*1-4 *flu*1-2 *flu*1-1

f

```
  1  MAALIRCCSSFSHTSGGQPPPRDKSRAPEIGKFATSIGYS
 41  VVRKPGDHPPFSKIIHSSSQPKERQGKGILQTPFASVGSL
                            *
 81  DKFSAFEGIGRLKLPVMAVLLTNSLQMATPLEALAAEICE
121  PESSMFSMPILLLVALIGATVGGLLARQRKGELQRLNEQL
                             V
161  RQINAALRRQAKIESYAPSLSYAPVGARIPDSEIIVEPKK
201  QELISKLKTGKTFLRNQEPEKAYTEFKIALELAQSLKDPT
                     *
241  EEKKAARGLGASLQRQGKYREAIQYHSMVLAISKRESEDS
                          V
281  GITEAYGAIADCYTELGDLEKAGKFYDTYIARLETD
```

g

flu

flu (compl.)

wt

Fig.8

FLU cDNA sequence

```
  1  ATGGCGGCGC TTATCCGGTG TTGCTCCTCC TTCTCTCATA CTTCCGGTGG
 51  CCAACCACCG CCGCGTGACA AGTCTCGAGC TCCAGAGATT GGAAAATTTG
101  CTACATCTAT TGGCTACTCA GTAGTCAGAA AACCTGGGGA TCATCCTCCT
151  TTCTCCAAAA TTATACATTC GTCTTCACAG CCTAAGGAGC GACAAGGAAA
201  GGGAATCTTA CAAACGCCAT TTGCCAGTGT GGGATCTCTT GACAAGTTTT
251  CAGCTTTTGA GGGAATTGGG AGGCTCAAAT TACCGGTGAT GGCAGTGTTG
301  TTGACAAACT CCCTTCAGAT GGCTACACCT CTTGAAGCTT TGGCTGCTGA
351  GATATGTGAG CCTGAGAGCT CCATGTTCAG CATGCCTATT TTGTTGCTTG
401  TTGCTCTTAT TGGAGCCACC GTTGGAGGGT TGCTTGCTCG GCAGAGGAAA
451  GGGGAGTTAC AGAGGTTGAA TGAACAACTA CGACAGATTA ACGCAGCTCT
501  TAGGAGACAA GCCAAAATCG AGTCTTATGC ACCAAGCTTG AGTTATGCTC
551  CAGTGGGAGC AAGAATCCCA GATAGTGAAA TCATTGTGGA ACCAAAGAAA
601  CAAGAGTTGA TTTCCAAGTT GAAAACAGGA AAAACCTTTC TTAGGAACCA
651  AGAACCAGAG AAAGCTTATA CAGAGTTCAA GATAGCTTTG GAACTTGCCC
701  AGAGTCTCAA AGACCCCACC GAAGAAAAGA AGGCTGCAAG AGGCCTAGGC
751  GCCTCGCTGC AGCGCCAAGG GAAGTATAGG GAAGCCATAC AGTATCACTC
801  CATGGTTCTA GCCATCTCAA AGAGGGAAAG TGAAGATTCT GGAATCACTG
851  AAGCATATGG AGCTATTGCA GACTGTTATA CCGAGCTGGG AGATCTCGAG
901  AAAGCTGGAA AGTTCTATGA TACGTACATT GCTCGGTTAG AGACTGACTG
951  A
```

Fig.9

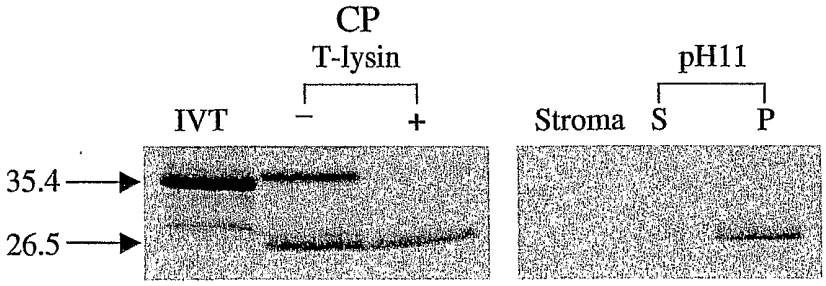


Fig.10

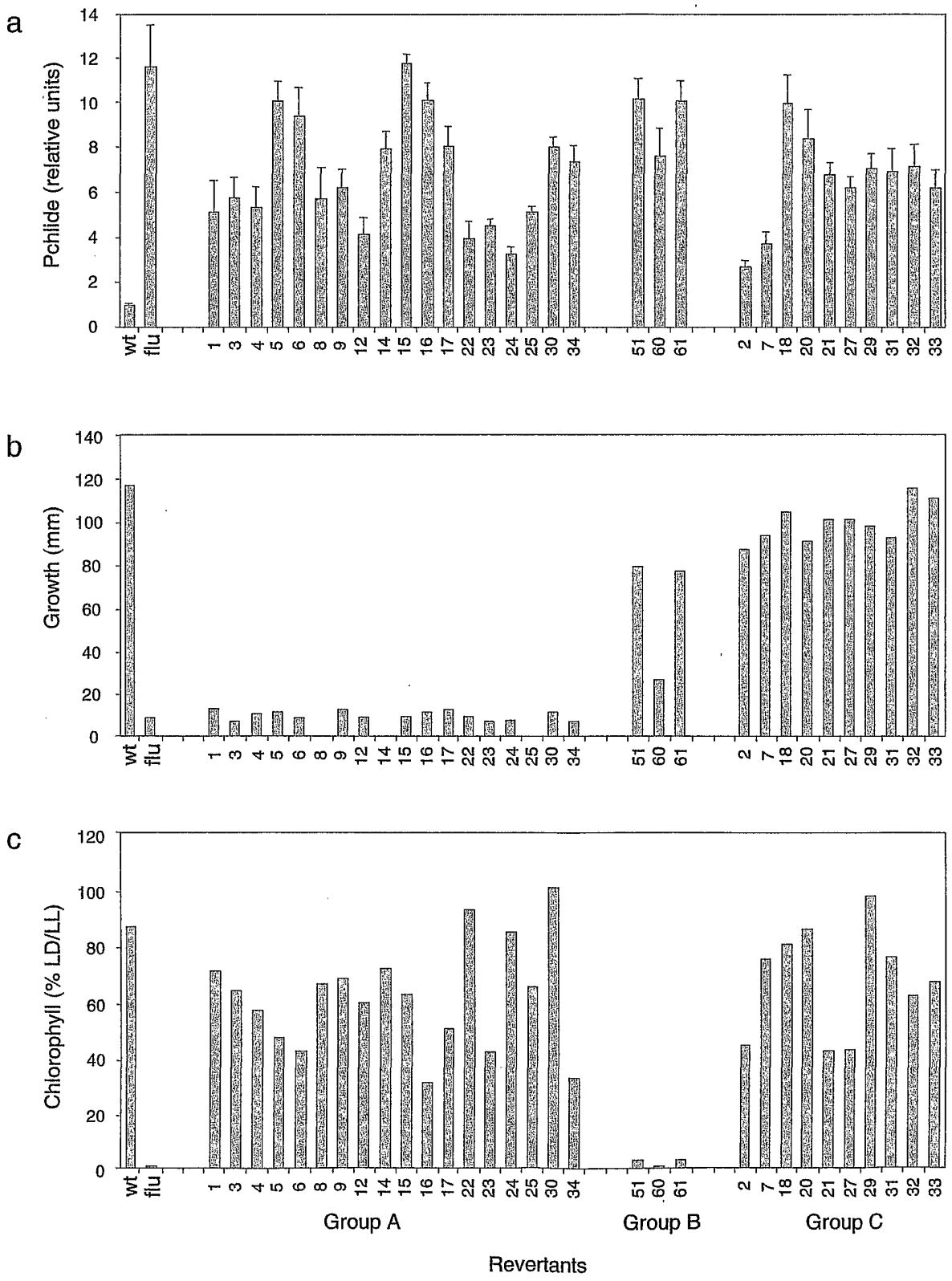

a
Pchlide (relative units)
0
2
4
6
8
10
12
14
b
Growth (mm)
0
20
40
60
80
100
120
140
c
Chlorophyll (% LD/LL)
0
20
40
60
80
100
120
wt
flu
1
3
4
5
6
8
9
12
14
15
16
17
22
23
24
25
30
34
51
60
61
2
7
18
20
21
27
29
31
32
33
Group A
Group B
Group C
Revertants

Fig.11

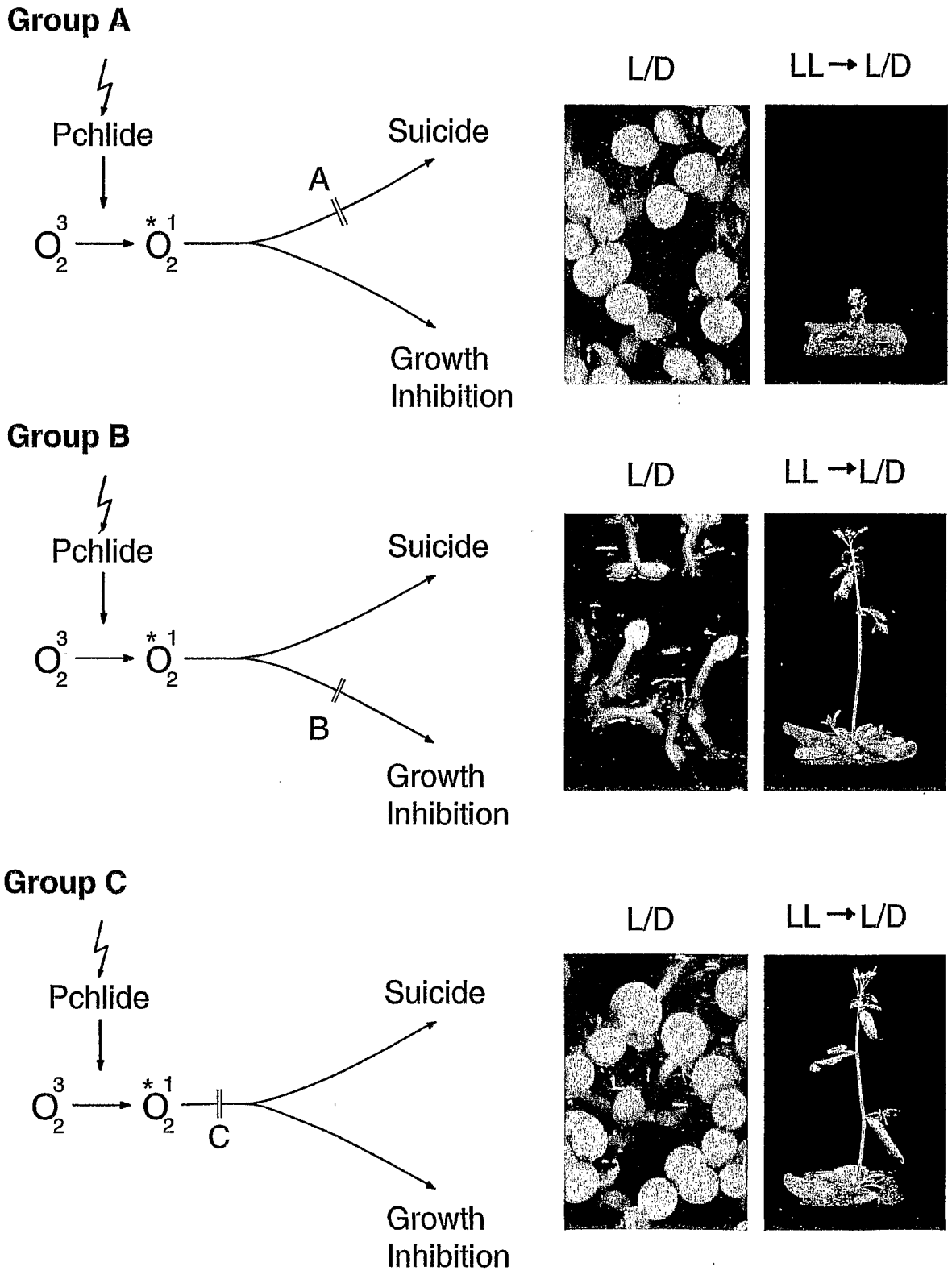
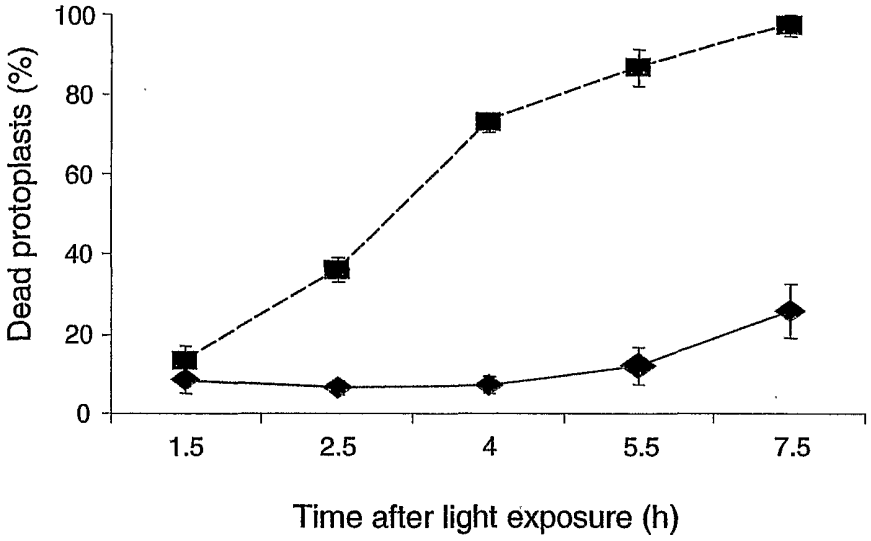
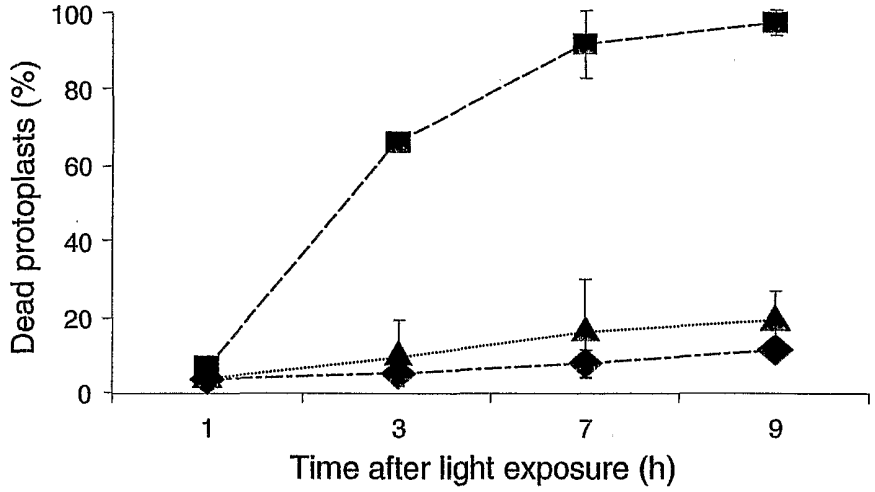

Group A
Pchlide
O_2^3
$^*O_2^1$
A
Suicide
Growth Inhibition
L/D
LL → L/D
Group B
Pchlide
O_2^3
$^*O_2^1$
B
Suicide
Growth Inhibition
L/D
LL → L/D
Group C
Pchlide
O_2^3
$^*O_2^1$
C
Suicide
Growth Inhibition
L/D
LL → L/D

Fig.12

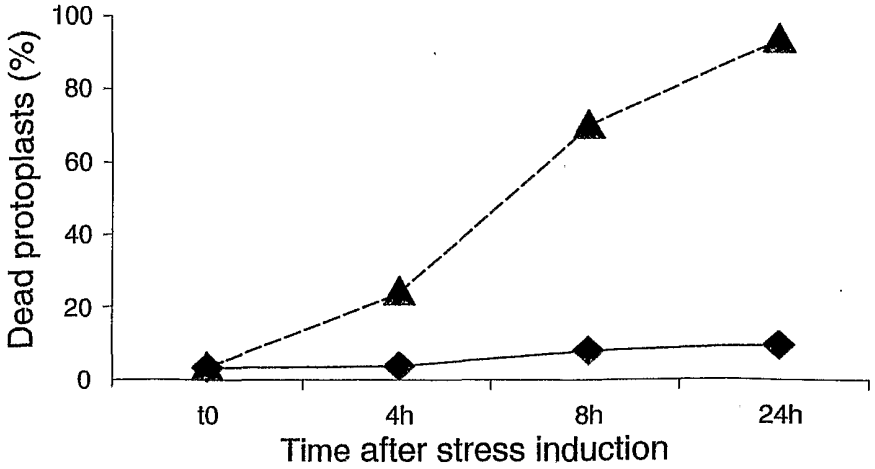
a


Dead protoplasts (%)
100
80
60
40
20
0
1.5
2.5
4
5.5
7.5
Time after light exposure (h)

b


Dead protoplasts (%)
100
80
60
40
20
0
1
3
7
9
Time after light exposure (h)

c


Dead protoplasts (%)
100
80
60
40
20
0
t0
4h
8h
24h
Time after stress induction

d

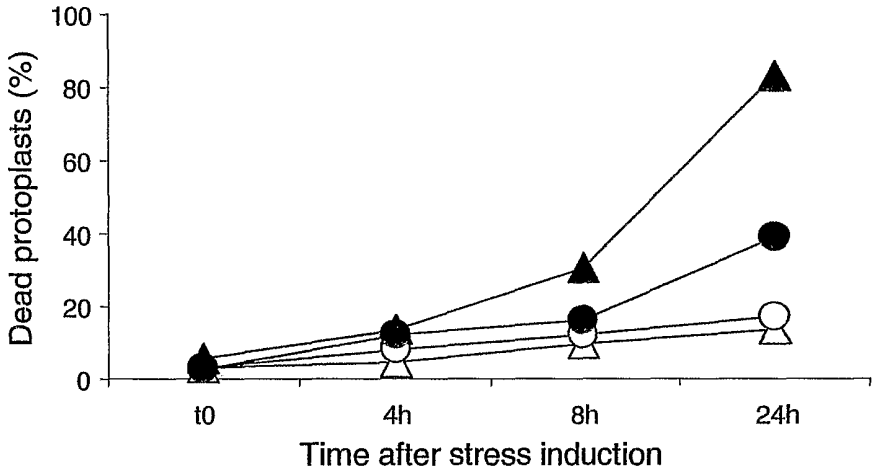

100
80
60
40
20
0
Dead protoplasts (%)
t0
4h
8h
24h
Time after stress induction

Fig.13