# Molecular Mechanism of Clomazone Resistance in *Oryza sativa* and *Echinochloa phyllopogon*

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# Molecular Mechanism of Clomazone Resistance in *Oryza sativa* and *Echinochloa phyllopogon*

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**Abbreviations**

TRJ, tropical japonica

TEJ, temperate japonica

DNA, deoxyribonucleic acid

BSM, bensulfuron-methyl

BPH, brown planthopper

CA, carbonic anhydrase

TSR, target-site resistance

NTSR, non-target-site resistance

QTLs, quantitative trait locus

BILs, backcross inbred lines

CSSLs, chromosome segment substitution lines

NILs, near-isogenic lines

MEP pathway, methylerythritol 4-phosphate pathway

FW, fresh weight

$I_{50}$, the 50% inhibitory concentration

P450, cytochrome P450 monooxygenase

ALS, acetolactate synthase

MS medium, murashige and skoog medium

DMSO, dimethyl sulfoxide

RNA, ribonucleic acid

cDNA, complementary deoxyribonucleic acid

PCR, polymerase chain reaction

ACCase, acetyl-CoA carboxylase.

ABT, 1-aminobenzo-triazole

PBO, piperonyl butoxide

BS, bispyribac-sodium

LC-MS/MS, liquid chromatography-tandem mass spectrometer

## INTRODUCTION

Rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and maize (*Zea mays* L.), which supply approximately half of the calories consumed by population of the world, are the most significant three cereals in the world, especially rice, comprising 23%-a tremendous ratio-of the daily calories in human diet (Khush, 2003). In 2000, over 50% of the world's people relied on rice as their major daily source of calories and protein. The global population, now at about 7 billion, is still increasing rapidly-approximately 8 million every year, and is expected to reach a population of 9.7 billion by midcentury and 11.2 billion by century's end based on the United Nations projects using their median scenario (United Nations, 2015). To satisfy the expanding food demands of the rapidly growing world population, 50% of crop grain productivity needs to be increased by 2050. A great many efforts were conducted to increase the crop productivity, particularly rice, such as modifying plant type, developing crop cultivars to be tolerant to abiotic environmental stresses and improving genes of crop cultivars (Khush, 2001, 2003).

There are 5 rice subgroups in the world-indica, temperate japonica (TEJ), tropical japonica (TRJ), aromatic, and aus which were classified based on cropping systems, geographical location, and weather patterns to which the subspecies is adapted (Garris et al., 2005; Belefant-Miller et al., 2010). The indica and japonica subspecies can be differentiated by phenol reaction, grain length and shape, cold-endurance, chlorate sensitivity, leaf color, apiculus hair length, and disease resistance (Oka, 1988; Ueno et al., 1990; Lu et al., 2005). Indica rice, which are non-sticky when cooked, are generally cultured in lowland of the tropics, while japonica rice, which are separated into TRJ and TEJ by the regions from which they were developed, are upland rices that are sticky. Aromatics and aus subspecies are genetically different from indica and japonica subspecies. The aromatics rice has a popcorn-like scent while the aus rice is drought-endurant and early maturing rice type (Garris et al., 2005).

Because of its importance as a principal source of food for the human population and the most compact genomes among the cereals, rice was chosen to be sequenced.

Rice is extensively accepted as a good model for the studies of monocot plants owing to its small genome-only about 3% as much DNA as wheat and about 20% as much DNA as maize (Somerville and Somerville, 1999). The research on rice have entered into a new period of functional genomics to the accompaniment of the publication of draft sequences of indica and japonica rice genomes (Goff et al., 2002; Yu et al., 2002) and accomplishment of sequencing chromosome 1 and 4 by International Rice Sequencing Project (IRGSP; Feng et al., 2002; Sasaki et al., 2002).

The major 4 aspects which affect crop production are insects, diseases, weeds, and abiotic stresses. Insects and diseases cause evaluated yield losses of approximate 25% in cereal crops annually (Khush, 2003). The progressed technology in rice transformation has made it feasible to produce new rice cultivars with improved tolerance to diseases, insects, and abiotic stresses by genetic engineering (Li et al., 1993; Xu et al., 1993, 1996). Fungal blast and bacterial blight diseases of rice, caused by *Pyricularia grisea* Sacc. and *Xanthomonas oryzae* pv. *Oryzae*, respectively, are two of the most destructive diseases in rice crop worldwide. It has been reported that three major genes-*Pi1*, *Piz-5*, and *Pita* which locate on chromosomes 11, 6, and 12, respectively, confer blast resistance in rice. The plants carrying the two- or three-gene combinations including *Piz-5* have enhanced blast resistance than when it is present alone in the Philippines and India (Hittalmani et al., 2000). Transgenic plants carrying *Xa26*-a rice gene which confers resistance to *X. oryzae* causing rice bacterial blight disease, showed enhanced resistance to bacterial blight compared with the donor line of the gene in both seedling and adult stages (Sun et al., 2004). Wen et al. (2003) also have been reported that some genes may have a phenotypic influence on fungal blast and bacterial blight resistance in rice. Transgenic rice plants carrying a truncated δ-endotoxin gene, *cryIAb* of *Bacillus thuringiensis* (B.t.), are more resistant to two major rice insect pests, striped stemborer (*Chilo suppressalis*) and leaffolder (*Cnaphalocrosis medinalis*) than wild-type control plants (Fujimoto et al., 1993). Small-scale field tests showed that the transgenic rice plants expressing the *CpTi* gene-a cowpea trypsin inhibitor gene, had significantly enhanced resistance to two species of rice stem borers, which are primary rice insect pests (Xu et al., 1996). Introgression of *Bph3* cluster-a locus in rice found more than 30 years ago, into susceptible rice cultivar by transgenic or marker-assisted selection strategies prominently increased resistance to both the

brown planthopper and the white back planthopper (Liu et al., 2015). Map-based cloning and characterization of the *BPH18* gene from wild-type rice confer resistance to brown planthopper (BPH) insect pest, which facilitate explanation of the molecular mechanism of BPH resistance and the identification of the novel alleles to fast track culturing BPH resistant rice cultivars (Ji et al., 2016).

There are many researchers reported that some genes of rice could confer multiple environmental stresses. Transgenic yeast (*Saccharomyces cerevisiae*) carrying the gene *rHsp90* of rice, displayed enhanced tolerance to NaCl, $Na_2CO_3$ and $NaHCO_3$; meanwhile, tobacco seedlings over-expressing *rHsp90* could endure salt solution as high as 200 mM NaCl, whereas seedlings of wild-type control couldn't. These results demonstrated that *rHsp90* plays an considerable role in various environal stresses (Liu et al., 2006). Yu et al. (2007) reported that transgenic Arabidopsis over-expressing *OsCA1* coding for carbonic anhydrase (CA) in leaves and roots of rice enhanced salt tolerance than wild-type plants at the seedling stage. Therefore, CA expression responds to abiotic stresses and is correlated to stress endurance in rice (Yu et al., 2007). Transgenic Arabidopsis expressing a rice heat stress transcription factor *OsHsfA2e*, enhanced tolerance to environmental stresses (Yokotani et al., 2008).

Rice output is influenced primarily by weeds in a greatest extent. To agriculturalists, weeds are defined as highly competitive plants that persistently adapt to cropping systems and cause crop loss and damage (Yuan et al., 2007). Weeds in crop fields will compete light, fertilizer, water with crops and transmit insects and diseases, reducing yields and grain quality. Weeds are a ubiquitous threat to agriculture and the average yield loss in rice because of weed competition are evaluated to vary between 30 and 60% which may reach to 94-96% with uncontrolled weed growth, especially herbicide resistant weed species (Chauhan and Johnson, 2011; Oerke, 2006; Pimentel et al., 2000).

It has been reported that weeds of rice are about 1800 species, those of the *Poaceae* and *Cyperaceae* are predominant. The relative abundance of weed species has been altered by the adoption of direct-seedling in rice crops. In particular, *Echinochloa* spp., *Cyperus difformis, Fimbristylis miliacea*, and *Ischaemum rugosum*

are widely adapted to conditions of direct-seeding rice (Rao et al., 2007). In recent decades, the prominent weed control heavily rely on chemical herbicide application which can inhibit weed growth efficiently. However, repeated usage of the herbicides with semblable mechanisms of action has imposed persistent selection for incremental resistance within/among weed species that had been susceptible (Haas and Streibig, 1982; Holt and Lebaron, 1989). Herbicide-resistant weed species have increased rapidly in recent years. *Echinochloa phyllopogon*, *Echinochloa oryzoides* and *Echinochloa crus-galli* are three main weeds of *Echinochloa* genus which is noxious in rice fields. The serious infestations of *Echinochloa* weeds cause large decreases in rice yields which is a severe threat to the population in the world (Fischer et al., 2000). In the late 1990s, the first resistant populations of *E. phyllopogon* were reported. After that, the resistance evolved quickly. To now, at least nine herbicides, which have different mode of actions, can not control the multiple-resistant *E. phyllopogon* populations (Fischer et al., 2000b; Osuna et al., 2002; Ruiz-Santaella et al., 2006; Bakkali et al., 2007; Yasuor et al., 2009, 2010, 2012). *E. oryzoides* and *E. crus-galli* also have been reported their resistance to some herbicides (Baltazar and Smith, 1994; Lopez-Martinez et al., 1997; Fischer et al., 2000; Im et al., 2009).

The indica rice variety is obtaining acceptance in USA rice germplasm and breeding programs for its disease-tolerant, and potentially weed-suppressive characteristics (Marchetti et al., 1998; Dilday et al., 2001a, 2001b; Gealy et al., 2005; Rutger et al., 2005; Yan and McClung, 2010; Gealy and Moldenhauer, 2012). However, japonica cultivars, which have high output potential and excellent grain quality, are not inherently weed-suppressive (Gealy et al., 2003; Moldenhauer et al., 2004; Gealy and Moldenhauer, 2012; Gealy and Yan, 2012), and are managed with a heavy reliance on herbicides to control weeds (Kendig et al., 2003). Because of its unique mode of action, clomazone is an excellent choice for herbicide-resistance weeds management in rice in the USA. However, its application is limited to long-grain varieties (e.g. indica cultivars) since middle- and short-grain varieties (e.g. japonica cultivars) are not tolerant to clomazone. The molecular of the tolerance mechanism of indica rice cultivars has not been reported yet.

The resistant mechanism of *Echinochloa* genus, which separates to target-site resistance (TSR) and non-target-site resistance (NTSR), have been reported in some

herbicides (Yasuor et al., 2009, 2010; Iwakami et al., 2012, 2014a, 2014b; Fischer et al., 2000b; Yun et al., 2005; Bakkali et al., 2007; Osuna et al., 2002). The progress of the research on the molecular level of the resistant mechanism of *Echinochloa* genus, however, developed slowly in the past decades. *E. phyllopogon*, which is a major weed in rice field and resistant to multiple-herbicide, has evolved resistance to clomazone after many years of repeated usage of herbicides other than clomazone. The molecular of resistant mechanism of *E. phyllopogon* to clomazone has not been reported yet, even though enhanced herbicide degradation by P450s and lower accumulation of the toxic metabolites in resistant plants endow multiple-herbicide-resistant *E. phyllopogon* with cross-resistance to clomazone were suggested (Yasuor et al., 2010).

To further understand the molecular mechanisms of tolerance/resistance of the herbicide clomazone, it is necessary to identify and analyze more genes that are involved in the plants. In this research, three rice subspecies and *E. phyllopogon* were selected as plant materials to conduct the experiments. Previously, Iwakami et al. (2014a, 2019) identified over-expression of two cytochrome P450 monooxygenase (P450) genes is associated with resistance to ALS inhibitors and ACCase inhibitors. Moreover, a similar mechanism has been suggested as the basis of resistance in clomazone (Yasuor et al., 2010). In this study, the results suggest that the over-expression of the two P450 genes are also involved in the clomazone resistance of *E. phyllopogon*. The functional characterization of other *CYP81A*s in *E. phyllopogon* by ectopic expression in Arabidopsis showed that the additional two genes also metabolize clomazone and an ALS inhibitor, underpinning the importance of *CYP81A* subfamily of P450 in the herbicide metabolism of plants. The results suggested orthologous genes in rice may explain the tolerance of indica rice to clomazone. To elucidate the molecular mechanism of the differential sensitivity among different rice varieties, I analyzed several cytochrome P450 genes in rice. In addition, I further located the chromosome region of clomazone resistance gene using genetic resources of short- and long-grain rice cultivars.

# CHAPTER 1

## Investigation of clomazone tolerance mechanism in *Oryza sativa*

**Abstract**

A herbicide, clomazone is converted into 5-keto clomazone in planta, and 5-keto clomazone inhibits deoxyxylulose 5-phosphate synthase catalyzing the first step of the methylerythritol 4-phosphate pathway, leading to the prevention of carotenoid biosynthesis in plants. Because of its unique mode of action, clomazone is an excellent choice for herbicide-resistance weeds management in rice fields in the USA. However, its application is limited to long-grain varieties since middle- and short-grain varieties are not tolerant to clomazone. The mechanism of clomazone tolerance/resistance in plants is largely unknown. Some researchers found that several P450 genes of rice confer resistance to some herbicides in Arabidopsis, then, in order to elucidate the molecular mechanism of the differential sensitivity among different rice varieties, I analyzed the several cytochrome P450 genes in rice. In addition, I located the chromosome region of clomazone resistance gene using genetic resources of short- and long-grain rice cultivars.

The whole seedlings of Nipponbare and Koshihikari rices were almost bleached at 1 μM of clomazone, while those of Kasalath rice were not affected. Kasalath rice stands approximately five times higher doses of clomazone. F1 seedlings of Nipponbare and Kasalath rices were sensitive to clomazone at 1 μM. Segregation of clomazone tolerance in F2 generation suggested that more than one locus are involved in clomazone tolerance in Kasalath rice. Transgenic Arabidopsis with one of the P450 genes from Kasalath rice showed reduced sensitivity to clomazone compared to wild-type one. However, no significant difference in clomazone sensitivity was observed between wild type and the P450 gene knocked out Kasalath rice, indicating the gene does not play an important role in clomazone tolerance in Kasalath rice. Several CSSLs with the same chromosome region on chromosome 5 showed partial tolerance to clomazone indicating that at least one gene locus which confer clomazone tolerance locate on chromosome 5. More research need to be performed to pinpoint the gene(s).

## 1. Introduction

The japonica rice cultivar Nipponbare and Koshihikari are widely cultured in Japan. Nipponbare rice is a standard cultivar and Koshihikari rice is a premium cultivar in Japan. Both of them present several finest traits of economic value, such as well eating quality, high tolerance to pre-harvest sprouting, and cool temperature endurance at the booting stage. Consequently, Nipponbare and Koshihikari rice subspecies have often been used as parental line to exploit new cultivars in Japanese rice breeding programs. However, the disadvantages of Nipponbare and Koshihikari rice subspecies are poor root system and low lodging tolerance (Morita et al., 1995; Kashiwagi et al., 2007). The indica rice cultivar-Kasalath is poor output with small 1,000-grains weight and a low proportion of filled grains (Ishimaru, 2003; Ishimaru et al., 2005; Kojima et al., 2005). Whereas, indica rice cultivars could suppress barnyardgrass, be resistant to diseases, and be tolerant to many herbicides. The genomic information of Nipponbare and Kasalath cultivars is already available which could be downloaded in The Rice Annotation Project Database (RAP-DB), especially Nipponbare cultivar which has higher accuracy (Madoka et al., 2008). Kasalath cultivar includes genes that could be used to improve multiple traits (e.g. root characters and disease or herbicide tolerance) in the Japanese premium rice-Nipponbare and Koshihikari cultivars. Quantitative trait locus (QTLs) analyses using near-isogenic lines (NILs), backcross inbred lines (BILs) and chromosome segment substitution lines (CSSLs) were always conducted to acquire genes that manage excellent traits (Ramos et al., 2016). CSSLs with these segments can be used for the simultaneous improvement of various characteristics.

Clomazone [2-(2-chlorobenzyl)-4,4-dimethyl-1,2-isoxazolidin-3-one], which can inhibit weeds effectively, is in the isoxazolidinones chetnical family (Ahrens, 1994). Clomazone is taken up by the roots and emerging shoots and is carried into the xylem to plant leaves (Senseman, 2007). After absorption by the plants, clomazone is known to be converted to an active form, 5-keto clomazone (Yasuor et al., 2008, 2010). 5-keto clomazone inhibits the deoxyxylulose 5-phosphate synthase (DXS) which belongs to the first committed step of the methylerythritol 4-phosphate (MEP) pathway, synthesizing isoprenoid pyrophosphate and dimethylallyl diphosphate, the precursors of isoprenoid molecules such as chlorophylls, carotenoids, and tocopherols

(Ferhatoglu and Barrett, 2006). Consequently, clomazone reduces or prevents accumulation of plastid pigments in susceptible species, which causes leaves of plants becoming bleached appearance (white, yellow, or pale-green in color) (Duke et al., 1991). Some rice cultivars have shown good tolerance to clomazone, even though substantial injury can occur under various circumstances, but did not affect rice yield in some cases. Furthermore, higher clomazone rates may increase rice yield by improving weed control (Webster et al., 1999; Zhang et al., 2005). The target enzyme of clomazone is DXS belonging to MEP pathway, which is a sole mode of action among herbicides. Because of this advantage, clomazone was selected to control herbicide-resistant weeds in rice field in the USA. However, since middle- and short-grain varieties (e.g. japonica cultivars) are not tolerant to clomazone, it can only be applied in long-grain varieties (e.g. indica cultivars) although the tolerance mechanism was not clear yet.

Cytochrome P450 monooxygenases (P450s), which are heme-thiolate proteins that contain a great and various group of isozymes and catalyze a wide variety of oxidative reactions in plants, animals and microorganisms, play a staple role in the Phase I metabolism of xenobiotics, particularly foreign chemicals-herbicides (Bolwell et al., 1994; Schuler, 1996; Chapple, 1998; Siminszky, 2006). The P450s, which play momentous roles in the process of growth and development, defense mechanisms, and interactions between plants and other creatures, participate in the biosynthesis of brassinosteroids, cyanogenic glucosides, jasmonic acid, flavonoids and many secondary compounds that work as plant defense agents in higher plants (Durst and O'Keffe, 1995; Schuler, 1996; Maneechote et al., 1997; Chapple, 1998; Cou and Kutchan, 1998). Some plant P450s which are involved in herbicide metabolism have been isolated and characterized. *CYP81A6* of rice can metabolize bentazon and sulfonylurea which belong to two different classes of herbicides (Pan et al., 2006; Zhang et al., 2007). *CYP71A11* and *CYP81B2* which were isolated from tobacco are involved in chlortoluron metabolism (Yamada et al., 2000). *CYP76B1* extracted from Jerusalem artichoke (*Helianthus tuberosus*) catalyzes rapid oxidative dealkylation of diverse phenylurea herbicides to produce nonphytotoxic metabolites (Didierjean et al., 2002). *CYP72A31* which isolated from Kasalath rice is involved in bispyribac-sodium (BS) and bensulfuron-methyl (BSM)-two acetolactate synthase (ALS)-inhibitors tolerance (Saika et al., 2014). Despite some P450 genes were

isolated and characterized, the ones that confer clomazone tolerance in rice varieties have not been reported yet.

In rice, there are more than 30,000 genes in the 12 chromosomes (IRGSP, 2005). QTLs are accumulated by crossing after narrowing down the chromosome regions containing desired loci. More importantly, rice CSSLs of Kasalath with Nipponbare or Koshihikari cultivars, in which chromosomal segments of the Kasalath cultivar substitute the corresponding endogenous segments in the genome of the Nipponbare or Koshihikari cultivars, together cover the entire genome and are available, respectively (Fig. 1). CSSLs are widely used and effectual in detecting excellent QTLs with small additive effects that are masked by QTLs with bigger additive effects in main populations (Shim et al., 2010; Ramos et al., 2016). Conducting the sensitivity test of CSSLs of Kasalath with Nipponbare or Koshihikari cultivars, the region which contains clomazone tolerant genes can be narrowed.

In this chapter, I analyzed several candidate P450 genes in rice and located the chromosome region of clomazone tolerance gene(s) using CSSLs of short- and long-grain rice cultivars to elucidate the molecular mechanism of the differential sensitivity among different rice varieties.

## 2. Materials and methods

### *2.1 Plant materials*

In this study, one long-grain cultivar, Kasalath, and two short-grain cultivars, Nipponbare (a standard Japanese cultivar) and Koshihikari (an elite Japanese cultivar), were used. F1 and F2 progenies of Nipponbare/Kasalath cultivars were generated by artificial cross pollination. CSSLs of Nipponbare/Kasalath cultivars and Koshihikari/Kasalath cultivars were provided by the Rice Genome Resource Center. A cytochrome P450 gene of Kasalath rice, *CYP81A6*, was knocked out by *CRISPR/Cas9* system in Kasalath rice. The seeds of transgenic Arabidopsis expressing *CYP72A31* were received from Saika Hiroaki (2014).

### *2.2 Generation of F1 and F2 progenies of Kasalath and Nipponbare cultivars*

Seedlings of Kasalath and Nipponbare cultivars were cultured in a growth chamber at 25°C under fluorescent light (approximately 300 μmol $m^{-2}$ $s^{-1}$) with a 12-h photoperiod (d 0). Before the seedlings flower, the stamen of Nipponbare rice seedlings were deleted. The next day, the mature stamen of Kasalath rice seedlings were selected to cross with the pistils which the stamen were deleted. After the crossed seeds grew mature, the sensitivity test to clomazone was conducted. At the same time, F1 seeds were cultured until maturation to collect F2 seeds.

### *2.3 Herbicide susceptibility of O. sativa*

The seeds of *O. sativa* were washed with 70% (v/v) ethanol for 30 s, sterilized twice with 50% (w/v) sodium hypochlorite, containing 0.1% (v/v) Tween 20, for 15 min, and washed three times in sterile water. The seeds were plated on moist filter paper in petri dishes and cultured in a growth chamber at 25°C under fluorescent light (approximately 300 μmol $m^{-2}$ $s^{-1}$) with a 12-h photoperiod (d 0). The germinated seeds were transferred to glass tubes which contain murashige and skoog (MS) medium (Murashige and Skoog, 1962) supplemented with clomazone (0, 0.3, 0.6, 1, 3, 6 or 10 μM) or BSM (0, 0.01, 0.1, 1, 10, 100 or 1000 μM) at the $4^{th}$ day. After 6 d, the shoots were excised and soaked in 100% (v/v) dimethyl sulfoxide (DMSO) (50 ml/g FW) in

a glass tube (Barnes et al., 1992; Hiscox and Israelstam, 1979; Monje and Bugbee, 1992). The tube was incubated at 25°C in the dark for 2 d. The concentration of extracted chlorophyll was calculated based on the absorbance value at 645 nm and 663 nm as described previously (Arnon, 1949). The absorbance was measured using Multiskan GO (Thermo-Fisher Scientific, Waltham, MA, USA). The 50% inhibitory concentration ($I_{50}$) was estimated by non-linear regression using the log-logistic model with the 'drc' package (Ritz et al., 2015) in R 3.3.3 (Team, 2016). The treatments of herbicides were replicated 4 times and the experiment was performed at least twice.

For root treatment at 2-leaf stage, the seeds of *O. sativa* were incubated in distilled water at 25°C in the dark for 2 d. The seeds were transferred to a growth chamber at 25°C under fluorescent light (approximately 300 µmol $m^{-2}$ $s^{-1}$) with a 12-h photoperiod (d 0). Plants were transferred to 250 ml vessels, containing 170 ml of 1/10 concentration of Kasugai nutrient solution (Ohta, 1970) at d 8 and of full concentration at d 11. The solution was replaced every three days. The roots of 2-leaf stage plants were dipped into clomazone solution (0, 10, 30, 60, 100 or 300 µM) for 24 h. At 6 d after the application, the shoots were excised and soaked in 100% (v/v) DMSO (50 ml/g FW) in a glass tube (Barnes et al., 1992; Hiscox and Israelstam, 1979; Monje and Bugbee, 1992). The chlorophyll content (four replicates) was measured as described above. The treatment of herbicide was replicated 4 times and the experiment was performed at least twice.

*2.4 P450 inhibitors' effect on herbicide susceptibility of O. sativa*

The seeds of *O. sativa* were washed with 70% (v/v) ethanol for 30 s, sterilized twice with 50% (w/v) sodium hypochlorite, containing 0.1% (v/v) Tween 20, for 15 min, and washed three times in sterile water. The seeds were plated on moist filter paper in petri dishes and cultured in a growth chamber at 25°C under fluorescent light (approximately 300 µmol $m^{-2}$ $s^{-1}$) with a 12-h photoperiod (d 0). The germinated seeds were transferred to glass tubes which contain MS medium supplemented with clomazone (0, 0.3, 0.6, 1, 3, 6 or 10 µM) or 5-keto clomazone (0, 0.01, 0.03, 0.06, 0.1 or 0.3 µM) and P450 inhibitors-ABT (100 µM) or PBO (1 mM) at the $4^{th}$ day. After 6 d, the shoots were excised and soaked in 100% (v/v) DMSO (50 ml/g FW) in a glass

tube (Barnes et al., 1992; Hiscox and Israelstam, 1979; Monje and Bugbee, 1992). The chlorophyll content (four replicates) was measured as described above. The treatments of herbicides were replicated 4 times and the experiment was performed at least twice.

### *2.5 Arabidopsis transformation*

Vector construction for *CYP81A5* (AK107231), *CYP81A6* (AK104825), and *CYP81A7* (AK059895) was performed as described previously (Iwakami et al., 2014a) with some modifications. Briefly, the full-length cDNA sequences of *CYP81A5*, *CYP81A6*, and *CYP81A7* were amplified by PCR using cDNA prepared from the shoot or root of Kasalath rice seedlings with the primers (Table 1). The amplicons were sub-cloned into pCAMBIA 1390 (Thermo-Fisher Scientific). The binary vectors were produced with In-fusion (Thermo-Fisher Scientific) from the sub-clones and pCAMBIA vector (Karimi et al., 2002). The binary vectors were transformed into *Agrobacterium tumefaciens* EHA105 as described previously (Hofgen and Willmitzer, 1988). The transformed *A. tumefaciens* was used for Arabidopsis (Col-0) transformation by the floral dip method (Clough and Bent, 1998). $T_3$ homozygous lines with a single copy of transgene were selected based on the segregation ratio against hygromycine at $T_2$ and $T_3$.

### *2.6 Real-time PCR for established transgenic Arabidopsis lines*

$T_3$ homozygous Arabidopsis lines were grown on MS medium (Murashige and Skoog, 1962) for 14 d . RNA was extracted from 4-5 plants using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using ReverTra Ace (Toyobo, Osaka, Japan). Real-time PCR was conducted with SYBR Green Realtime PCR Master Mix (Toyobo) on the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the primers listed in (Table 1). Data were normalized with *GAPDH* using the ΔΔCT method (Schmittgen and Livak, 2008). The RNA extraction of every line was replicated 3 times.

### *2.7 Herbicide susceptibility of transgenic Arabidopsis*

The seeds of transgenic Arabidopsis were washed with 70% (v/v) ethanol for 30 s, sterilized with 0.1% (w/v) sodium hypochlorite, containing 0.1% (v/v) Tween 20, for 3 min, and washed three times in sterile water. The sterilized seeds were placed on solid MS medium in petri dishes supplemented with BS (0, 0.01, 0.1 or 1 μM), clomazone (0, 0.03, 0.1, 0.3, 1, 3, 10 or 30 μM), 5-keto clomazone (0, 0.01, 0.03, 0.1, 0.3, 1 or 3 μM) or BSM (0, 1, 3, 10, 30, 100, 300 or 1000 nM). The dishes were kept in a growth chamber at 22°C under fluorescent light (approximately 70 μmol $m^{-2}$ $s^{-1}$) and a 12-h photoperiod for 14 d. The position of each dish was changed every 1-2 d. The chlorophyll content of 10 seedlings (four replicates) was measured at 14 d after the treatment as described above. The treatments of herbicides were replicated 4 times and the experiment was performed at least twice.

### *2.8 Yeast transformation and expression of CYP81A6*

*CYP81A6* derived from the Kasalath rice seedlings was expressed together with a gene encoding its redox partner, cytochrome P450 reductase (*ATR1*) from *Arabidopsis thaliana* (L.) Heynh. in budding yeast. *CYP81A6* was synthesized and the codon was optimized to be expressed in yeast by the GenPart DNA fragment service (GenScript, Piscartway, NJ, USA). DNA fragment of *CYP81A6* was re-amplified by PCR using the PrimeSTAR MAX DNA polymerase (Takara, Shiga, Japan) and appropriate primer sets (Table 1). The subsequent processes were conducted as described previously (Iwakami et al., 2018).

## 3. Results

### *3.1 Clomazone susceptibility of O. sativa*

The seedlings of Nipponbare rice was more affected by clomazone application at same concentration compared with that of Kasalath rice (Seedlings of Koshihikari rice had similar phenotype to clomazone with that of Nipponbare rice). The most prominent difference between Nipponbare and Kasalath rice seedlings was observed in leaf color (Fig. 2A & B) compared with fresh weight of the seedlings, especially at low concentrations (data not shown).

Chlorophyll content of the seedlings of Nipponbare rice was beginning to decrease at 0.6 μM of clomazone. One micromolar application resulted in white color of almost the whole seedlings of Nipponbare rice while that in Kasalath rice was not affected at all. $I_{50}$ for Nipponbare and Kasalath rice subspecies were 0.74 μM and 3.55 μM, respectively, resulting in $I_{50}$ ratio (K/N) of them was about 4.8 (Fig. 2C). Similar $I_{50}$ ratio (K/N=4.2) was obtained for the dose response of Nipponbare and Kasalath rice subspecies at 2-leaf stage, although $I_{50}$ for both cultivars were higher than germinated seeds, 11.78 μM and 49.48 μM for Nipponbare and Kasalath plants, respectively (Fig. 2D).

### *3.2 Segregation analyses of herbicide susceptibilities in Kasalath and Nipponbare rice varieties*

Inheritance of clomazone tolerance was assessed in the progeny of crosses between Kasalath and Nipponbare plants. F1 seeds were obtained by crosses of Kasalath and Nipponbare rice subspecies-the pollen donor is Kasalath rice and the pistil donor is Nipponbare rice. At 1 μM of clomazone, the whole seedlings of 11 F1 were bleached which were similar as that of Nipponbare rice (Fig. 3A & B). This result indicated that the genes which confer clomazone resistance of Kasalath rice are recessive genes.

Tolerance segregated in the F2 population on media containing 1 μM clomazone, concentration on which the leaves of Nipponbare rice seedlings became

white (Fig. 3A). The susceptibility was assessed by measuring the chlorophyll content of the leaves 6 d after clomazone application. For clomazone tolerance, segregation approached a 14:14:56 ratio (tolerant:intermediate:sensitive), which was not a 1:2:1 ratio (Fig. 3C), suggesting that the tolerance to clomazone was under the control of more than one gene loci.

How can we identify the molecular genes which are involved in clomazone tolerance in rice? Based on previous reports, some P450 genes of rice which confer herbicides resistance in transgenic Arabidopsis, attracted my attention. But, it is better to check whether P450s are involved in herbicide tolerance in rice firstly. Then, herbicide susceptibility of Kasalath and Nipponbare cultivars between treated and non-treated P450 inhibitors were performed.

*3.3 Herbicide susceptibility of Kasalath and Nipponbare cultivars between treated and non-treated P450 inhibitors*

It is reported that the toxicity of clomazone to rice or other crops can be reduced by organophosphate insecticides (Jordan et al., 1998; Culpepper et al., 2001; Ferhatoglu et al., 2005). Applying Kasalath and Nipponbare rice seedlings with known P450 inhibitors-100 μM 1-aminobenzo-triazole (ABT) or 1mM piperonyl butoxide (PBO) reduced their susceptibility to clomazone (Fig. 4A & B). The average protective effect did not differ between inhibitors both for Kasalath and Nipponbare plants, but was stronger on Nipponbare than Kasalath plants (Fig. 4A & B). These results indicated that clomazone toxicity maybe preceded by the oxidative conversion of P450s to a herbicidal active metabolite, for instance, 5-keto clomazone.

Responses to 5-keto clomazone were also evaluated, because this compound may be the primary toxic metabolite resulting from the bioactivation of clomazone (Ferhatoglu and Barrett, 2006). Like clomazone, Nipponbare rice seedlings also had higher susceptibility to 5-keto clomazone than Kasalath rice seedlings (Fig. 5A & B). However, the effects of P450 inhibitors on responses to 5-keto clomazone were different to those observed with clomazone. Only ABT protected Kasalath rice seedlings from 5-keto clomazone injury, while PBO did not affect 5-keto clomazone

toxicity (Fig. 5A). For Nipponbare rice seedlings, both ABT and PBO marginally protected them from 5-keto clomazone injury (Fig. 5B). These results suggested that P450s play a different role in regulating responses to clomazone and 5-keto clomazone in Kasalath and Nipponbare rice varieties. Since ABT protected Kasalath from 5-keto clomazone injury (Fig. 5A), moreover, both ABT and PBO marginally protected Nipponbare from 5-keto clomazone injury (Fig. 5B), it would be logical to infer that a P450-mediated metabolism of 5-keto clomazone might induce the generation of yet other more active intermediates in these plants.

The results suggested that P450s are involved in clomazone tolerance in rice, inducing my interest to analyze some candidate genes based on previous reports (Saika et al., 2014; Pan et al., 2006).

*3.4 Clomazone susceptibility of Arabidopsis transformed with CYP72A31*

Saika et al. (2014) reported that *CYP72A31* from Kasalath rice variety is involved in tolerance toward two ALS inhibitors, BS and BSM. Then, one query "whether *CYP72A31* confers clomazone tolerance in Kasalath rice variety" was established. After I received the seeds of transgenic Arabidopsis expressing *CYP72A31*, their sensitivity to BS and clomazone was performed.

At the concentration 0.1 μM of BS, the transgenic lines grew well while wild-type Arabidopsis could not grow, even germinate at all, which was consistent with previous report. However, the transgenic seedlings had identical response to 0.1 μM of clomazone with wild-type ones (Fig. 6), suggesting that *CYP72A31* did not confer clomazone resistance in transgenic Arabidopsis. Therefore, *CYP72A31* will not be involved in tolerance in Kasalath rice variety.

*3.5 Gene structures and expression level of CYP81As in rice*

A rice P450 gene-*CYP81A6*, confers resistance to two different classes of herbicides including bentazon and sulfonylurea (Pan et al., 2006). It is reported that enhanced detoxification-based herbicide resistance by P450s is particularly difficult to control, because it can evolve resistance to multiple, and chemically unrelated classes

of herbicides (Siminszky, 2006). Therefore, whether *CYP81A6* confers clomazone resistance is very interesting to investigate.

Except *CYP81A6*, there are other 3 *CYP81A* genes-*CYP81A5*, *CYP81A7* and *CYP81A8* in rice genome. Because the genome sequence of Kasalath and Nipponbare rice subspecies are available on RAP-DB, the sequences were downloaded. After that, the gene structures were established which showed in Fig. 7A. At the same time, an additional stop codon at the first exon of *CYP81A8* of Kasalath rice was found. RNA was extracted from the seedlings of Kasalath and Nipponbare cultivars and then reverse transcription was conducted to get cDNA. cDNA was used as the template to conduct sequencing to confirm the sequence of *CYP81A8* of Kasalath rice. Finally, *CYP81A8* of Kasalath rice was confirmed as a pseudogene, demonstrating that the gene has no function and would not be involved in herbicide tolerance in Kasalath rice variety.

The expression level of *CYP81A5*, *CYP81A6* and *CYP81A7* were compared between Kasalath and Nipponbare rice subspecies. Interestingly, *CYP81A6* expressed a higher level in Kasalath leaf than that in Nipponbare leaf. Nevertheless, the expression level of *CYP81A5* and *CYP81A7* were very low in both rice species, especially that of *CYP81A5* (Fig. 7B). Higher expression level of *CYP81A6* may perform an important role in Kasalath rice variety, but it does not represent *CYP81A5* and *CYP81A7* have no function. *CYP81A6* also expresses different level at different rice organs (Pan et al., 2006).

Finally, *CYP81A5*, *CYP81A6* and *CYP81A7* were amplified and introduced to Arabidopsis (*Arabidopsis thaliana*; ecotype Columbia-0) under the control of the Cauliflower mosaic virus 35S promoter. After selected T3 homozygous lines, I conducted their susceptibility to clomazone and BSM, respectively.

*3.6 Clomazone susceptibility of Arabidopsis transformed with CYP81A6*

Five homozygous lines were selected for comparison of *CYP81A6* transcript level (Fig. 8A). Transcript accumulation levels of the transgene were different among

the five lines. Two lines-#20 and #21 were selected to conduct the dose response to clomazone. Clomazone susceptibility differed between the two lines-the line with higher transcript level has higher resistant level (Fig. 8B & C). The $I_{50}$ for line #20, line #21 and wild type are 0.23 μM, 0.073 μM and 0.062 μM, respectively. The resistant index between line #20 and line #21 with wild type are 3.71 and 1.18, respectively. The clomazone susceptibility was correlated to the transcription level of *CYP81A6* in the transgenic lines. Therefore, *CYP81A6* conferred a decrease in clomazone susceptibility to transgenic Arabidopsis depending on its transcript abundance.

*3.7 Herbicide susceptibility of Arabidopsis transformed with CYP81As*

The results of transformed Arabidopsis with *CYP81A6* suggested that *CYP81A* P450s metabolize not only ALS inhibitors but also clomazone. *O. sativa* possesses at least 4 *CYP81A*s, including *CYP81A6*, one of which is frameshift-type pseudogenes (Fig. 7A). Here, I compared the susceptibility of Arabidopsis transformed with different *CYP81A*s (except for the one pseudogene) to BSM and clomazone. Lines with the highest expression level of the transgenes among five independently transformed lines were selected for this study (Fig. 9A & B). The 1 nM BSM application resulted in the growth inhibition of wild-type Arabidopsis, whereas the 3 nM BSM application in the plant death (Fig. 9D). Arabidopsis transformed with *CYP81A6* showed resistance to BSM application up to 1,000 nM illuminating *CYP81A6* confers resistance to BSM which are consistent with previous reports (Pan et al., 2006; Saika et al., 2014). Thus, the resistant index was more than 1,000. Arabidopsis transformed with *CYP81A7* also exhibited decreased susceptibility to BSM, but the resistance level was lower than that of those transformed with *CYP81A6*, even the resistant index was about 1000 which was also very high (Fig. 9D).

However, *CYP81A7* didn't exhibit resistance to clomazone at all (Fig. 9E). Although *CYP81A6* in Arabidopsis confers resistance to clomazone, the resistant index is 3.71 which is not as high as that of BSM (more than 1000) (Fig. 8). *CYP81A5* didn't confer resistance to both BSM and clomazone in Arabidopsis (Fig. 9D & E).

*3.8 Susceptibility of Arabidopsis transformed with CYP81As to 5-keto clomazone*

Subsequently, I investigated the susceptibility of the three Arabidopsis lines (*CYP81A5*, *CYP81A6* and *CYP81A7*) to 5-keto clomazone, an active form of clomazone. The 0.03 μM 5-keto clomazone application resulted in the total bleaching of wild-type Arabidopsis but did not affect the growth of Arabidopsis transformed with *CYP81A6* (Fig. 10). The transgenic line was severely affected at 0.1 μM of 5-keto clomazone application. Consistent with clomazone application, only Arabidopsis transformed with *CYP81A6* exhibited resistance.

### *3.9 Clmazone and 5-keto clomazone metabolic functions of CYP81A6*

To evaluate the ability of *CYP81A6* to metabolize clomazone or 5-keto clomazone, recombinant *CYP81A6* protein was produced using a yeast strain (*Saccharomyces cerevisiae*) expression system that carried the Arabidopsis NADPH-cytochrome P450 reductase gene *ATR1* (Pompon et al., 1996).

For the metabolis assay, clomazone or 5-keto clomazone was added to the yeast cultural media. After 24 hours, the media were analyzed by LC-MS/MS. There was no peak detected in the media of yeast expressing *CYP81A6*, same as in the empty vector control. The reasons for detecting metabolites unsuccessfully maybe due to the expression level of *CYP81A6* is too low to metabolize clomazone or the quantity of some metabolites are too small to be detectable.

### *3.10 Herbicide susceptibility of mutant Kasalath rice*

The cytochrome P450 gene of Kasalath rice, *CYP81A6*, was knocked out by CRISPR/Cas9 system. Clomazone and BSM susceptibility of wild-type Kasalath rice, wild-type Nipponbare rice, and mutant Kasalath rice were conducted subsequently. At 1 μM of clomazone, the whole seedlings of wild-type Nipponbare rice became white while that of wild-type Kasalath and mutant Kasalath rice were almost not affected at all (Fig. 11A & B), demonstrating the gene does not play an important role in clomazone tolerance in Kasalath rice.

Kasalath rice knocked out *CYP81A6* was more susceptive to BSM than wild-type Kasalath rice, more importantly, mutant Kasalath rice had higher susceptibility than wild-type Nipponbare rice. At 0.1 μM of BSM, the growth of mutant Kasalath rice seedlings were inhibited significantly, while that of wild-type Kasalath and Nipponbare rice were not affected at all (Fig. 12A). The seedlings of wild-type Nipponbare rice were inhibited seriously when the concentration of BSM increased to 10 μM (Fig. 12B). The $I_{50}$ ratio between wild-type and mutant Kaalath was about 364 (Fig. 12C). These results illustrated that *CYP81A6* is involved in BSM tolerance in rice.

*3.11 Clomazone susceptibility of CSSLs*

54 and 39 CSSLs of Nipponbare/Kasalath cultivars and Koshihikari/Kasalath cultivars were used to evaluate their susceptibility to 1 μM of clomazone, concentration on which Kasalath and Nipponbare/Koshihikari can be differentiated significantly. There are more than ten lines showed partial tolerance to clomazone at 1 μM. However, because of the limited number of the seeds for every line of CSSLs-only 20 seeds, it is better to culture the lines which are partial tolerant to 1μM of clomazone to collect more seeds, then conduct dose response to confirm their susceptibility.

After I collected enough seeds, dose response of the media tolerant CSSLs were performed. Finally, two seedlings of CSSLs-SL22 and SL24 of Nipponbare/Kasalath cultivars which showed media tolerance between Kasalath and Nipponbare seedlings were found. $I_{50}$ of SL22 and SL24 were 1.22 μM and 1.40 μM, respectively (Fig. 13A), which were lower than that of Kasalath rice, but higher than that of Nipponbare rice. SL22 has the whole chromosome 5 of Kasalath rice variety, while SL24 only contains one segment of that-between marker R2289 and C1230. Because SL23 and SL25, which involve one segment of chromosome 5, respectively, were sensitive to clomazone, the tolerant genes should be located between marker R2289 and C1230. As supplementary data, the dose response of the lines-SL212, SL213 and SL214 of the other group CSSLs of Koshihikari and Kasalath cultivars, which also contain one segment of chromosome 5, were performed. The results showed that seedlings of SL212 and SL214 were media tolerant to clomazone and $I_{50}$ of SL212 and SL214

were 1.32 μM and 1.33 μM, which were similar to that of SL22 and SL24 (Fig. 13B), while SL213 was sensitive. Compared the results of the two CSSLs groups, the tolerant gene loci were suggested to locate in the region between marker R2289 and C128. It is interesting to continue to conduct research of this segment.

## 4. Discussion

The results showed that Kasalath rice variety are tolerant to clomazone and BSM (Fig. 2 & 12), actually also to BS (Saika et al., 2014), suggesting Kasalath rice variety has multiple herbicides tolerance which is a very important advantage for weed management in rice field. Therefore, conducting the survey of the tolerant genes become more interesting. It has been reported that *CYP81A6* in Arabidopsis exhibit resistance to both bentazon and BSM, while *CYP72A31* confers resistance to both BS and BSM (Pan et al., 2006; Saika et al., 2014), explaining that the tolerance ratio of Kasalath and Nipponbare rice seedlings to BSM (higher than 10) was higher than that to BS (lower than 10). However, the tolerance genes in Kasalath plants to clomazone have not been identified yet. A detailed survey of the tolerant genes in Kasalath rice variety might be a useful approach to understand the mechanism of defense response in plants.

An artificial cross between Kasalath and Nipponbare cultivars was conducted to collect their progeny F1 and F2. Eleven F1 seedlings were sensitive to 1 μM of clomazone, indicating the tolerant genes are recessive genes, meanwhile F2 ratio illuminated more than one gene loci manage Kasalath rice tolerance to clomazone (Fig. 3). These results demonstrated that the tolerance to clomazone in Kasalath rice variety is much more complicated.

*CYP72A31* confers resistance to BS and BSM in Arabidopsis, but does not to clomazone, while *CYP81A6* confers resistance to bentazon, BSM and also clomazone (Fig. 6 & 8 & 9), explaining P450 genes play different role in managing responses to herbicides. Barrett et al. (1997) also reported that a presumed P450 gene is genetically linked to tolerance in maize against three herbicides which have different mode of action. As far as we know, *CYP81A6* and *CYP72A31* are the sole two cytochrome P450 genes involved in non-target herbicides tolerance in rice (Saika et al., 2014; Pan et al., 2006). There are many P450 genes are involved in rice genome, urging us to perform more effort to search the herbicide tolerance genes.

Kasalath rice variety were confirmed to be tolerant to several herbicides (Saika et al., 2014, Fig. 11 & 12), illustrating herbicides tolerant genes must be somewhere

in Kasalath genomes. P450 inhibitors protected both Kasalath and Nipponbare rice varieties from clomazone injury, indicating that clomazone toxicity to rice is fully exerted after metabolic monooxidation to more toxic metabolites, like that in *E. phyllopogon* (Yasuor et al., 2008). Moreover, P450 inhibitors slightly mitigated 5-keto clomazone (the presumed active metabolite of clomazone) injury to Nipponbare rice variety and only one P450 inhibitor protected Kasalath rice variety from 5-keto clomazone injury, demonstrating that after clomazone inverting to 5-keto clomazone, P450 genes catalyze 5-keto clomazone to some more toxic intermediates. More interestingly, the protection level by P450 inhibitors for clomazone is lower in Kasalath rice compared that in Nipponbare rice (Fig. 4 & 5), illuminating two possibilities: (1) In Kasalath rice variety, clomazone might convert to some metabolites which are less toxic than that in Nipponbare rice variety; (2) Clomazone tolerance may not strongly depend on enhanced P450 oxidation detoxification. Einaggar and Liu had been reported that esterases hydrolyze 5-keto clomazone led to the formation of its hydroxyl acid and subsequent glycosidic conjugation in soybean and some microorganisms (Einaggar et al., 1992; Liu et al., 1996). The contribution of other detoxification mechanisms to clomazone in Kasalath rice variety needs further investigations.

CSSLs are often used to select excellent QTLs, which is one of the most popular and useful ways (Shim et al., 2010; Ramos et al., 2016). In this study, 54 CSSLs of Kasalath/Nipponbare cultivars and 39 that of Kasalath/Koshihikari cultivars were used to examine the susceptibility to clomazone. Finally, the tolerant gene loci was focused on the region of chromosome 5-between marker R2289 and C128. Although there are still many genes involved in this segment, the shortening will decrease the difficulty to search the tolerant genes remarkably, especially P450 genes involved in this part. More further investigations need to be performed to find the interesting genes.

## 5. Summary

More than one gene loci, which are recessive genes, determine clomazone tolerance in Kasalath rice variety which has lower susceptibility to clomazone than Nipponbare rice variety. P450 inhibitors can protect Kasalath and Nipponbare rice varieties from clomazone injury, elucidating that clomazone toxicity may be preceded by P450s' oxidative conversion to a herbicidal active metabolite. *CYP81A6*, which has a higher expression level in Kasalath rice shoots than that in Nipponbare rice shoots, does not play an important role in clomazone tolerance in Kasalath rice, however, it is involved in BSM tolerance in rice. Further, the genes, which are involved in clomazone tolerance in Kasalath rice, are suggested locating in the segment of chromosome 5--between the marker R2289 and C128.

Table1 Primers used in this chapter

| Gene | Forward primer | Reverse primer |
|---|---|---|
| | **Sequencing of *CYP81A8*** | |
| *CYP81A8* | 5'-TAAGCCGGGCCAATGC-3' | 5'-CGTAGTTGGCACTGCCTAGA |
| | **Real-time PCR in *Oryza sativa*** | |
| *CYP81A5* | 5'-CACGAGCAATGGCAAGAAC-3' | 5'-CTCCTCGACGACCTGCTTGT-3 |
| *CYP81A6* | 5'-TGTAGCTATCCTCTTCTTGCTCCA-3' | 5'-CGCCGTTGAACGAGACCA-3' |
| *CYP81A7* | 5'-GAGACGACATGCCTCATCTTAG-3' | 5'-TGGCCTCCAACGGGACAG-3' |
| *rRNA* | 5'-CGGCTACCACATCCAAGGAA-3' | 5'-TGTCACTACCTCCCCGTGTCA |
| | **Real-time PCR in Arabidopsis** | |
| *CYP81A5* | 5'-ACATGTCCATGGAAGCCCAG-3' | 5'-GATCAGTCGACGGAGGAAC |
| *CYP81A6* | 5'-GAAGATCCTCGCCGCTGTAA-3' | 5'-TCGCCGTTAGAGCTGTGATC |
| *CYP81A7* | 5'-ACTTACTATTTCCGTAACCATGGA-3' | 5'-GGCTTGTCGATGAGGTGGA |
| *GAPDH* | 5'-TTGGTGACAACAGGTCAAGCA-3' | 5'-AAACTTGTCGCTCAATGCAAT |
| | **Full-length amplification for Arabidopsis transformation** | |
| *pCAM81A5-FLAG-F1* | 5'-TACAATTACAGTCGAATGGACTACAAAGACGATGACGACAAGGATAAGGCCTACAT | |
| *81A5_Kt-R2* | 5'-CGAGCAAAGAGCCGTGATC-3' | |
| *81A5_Kt-F3* | 5'-ACGGCTCTTTGCTCGAACTTGTTAGGAGCAGGAACAGA-3' | |
| *pCAM81A5-R1* | 5'-CCGCTTTACTTGTACTCAGAGCTCGCGAAGAACAC-3' | |
| *pCAM81A7-FLAG-F1* | 5'-TACAATTACAGTCGAATGGACTACAAAGACGATGACGACAAGGATAAGGCCTACAT | |
| *81A7_Kt-R2* | 5'-CGCACAAAGATTCACGATC-3' | |
| *81A7_Kt-F3* | 5'-GTGAATCTTTGTGCGGCATTATTTGCTGCTGGAAC-3' | |
| *pCAM81A7-R1* | 5'-CCGCTTTACTTGTACTCACAAGAGTTCCCGAAAAAC-3' | |
| *pCAM81A6-F1* | 5'-TACAATTACAGTCGAATGGACTACAAAGACGATGACGACAAGGATAACGCCTACAT | |
| *pCAM81A6-R1* | 5'-CCGCTTTACTTGTACTCAGACGAGCTCGCGAAGA-3' | |
| | **Vector construction for yeast expression** | |
| GbSc-81A6syn-Fw | 5'-cccgggtaccaaaaaaATGGATAACGCCTACATTA-3' | |
| GbSc-81A6syn-Rw | 5'-atcccccgcggaattcTTCTTCGCGAGCTCGTCTGA-3' | |

Note: Lower case represents nucleotides for directional cloning with pENTR-D-TOPO; Red case represents that overlap with the digested pCAMBIA1390-sGFP for the In-Fusion reaction; Blue case represents the sequence of Flag.

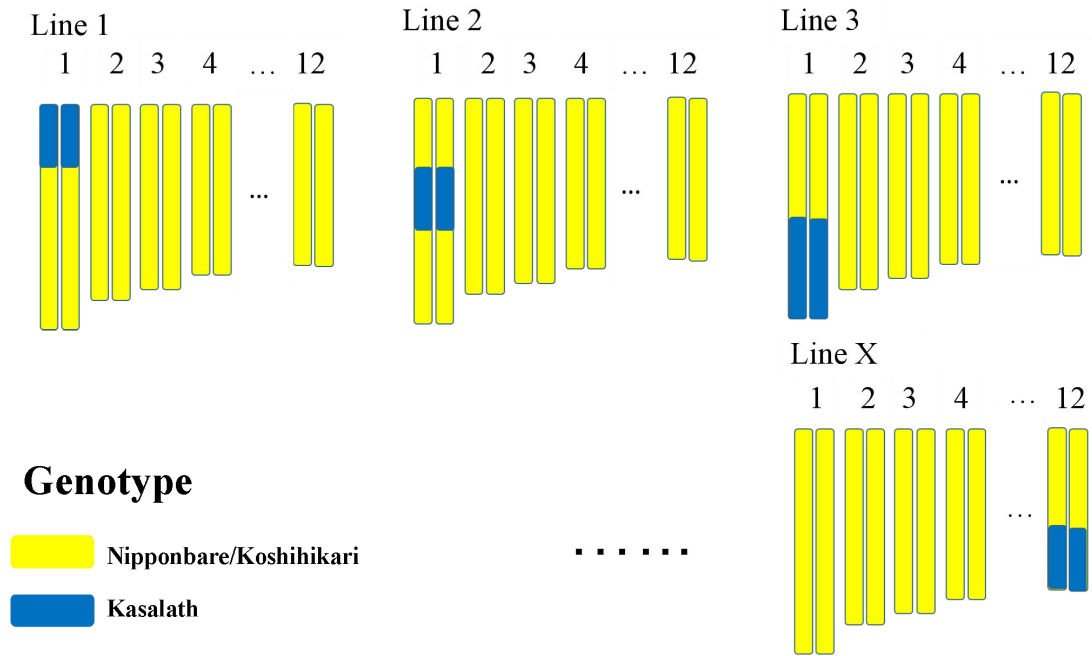


**Fig. 1.** Chromosome segment substitution lines (CSSLs) of Kasalath and Nipponbare/Koshihikari rice subspecies. Yellow, Nipponbare/Koshihikari chromosome; Blue, Kasalath chrmosome.

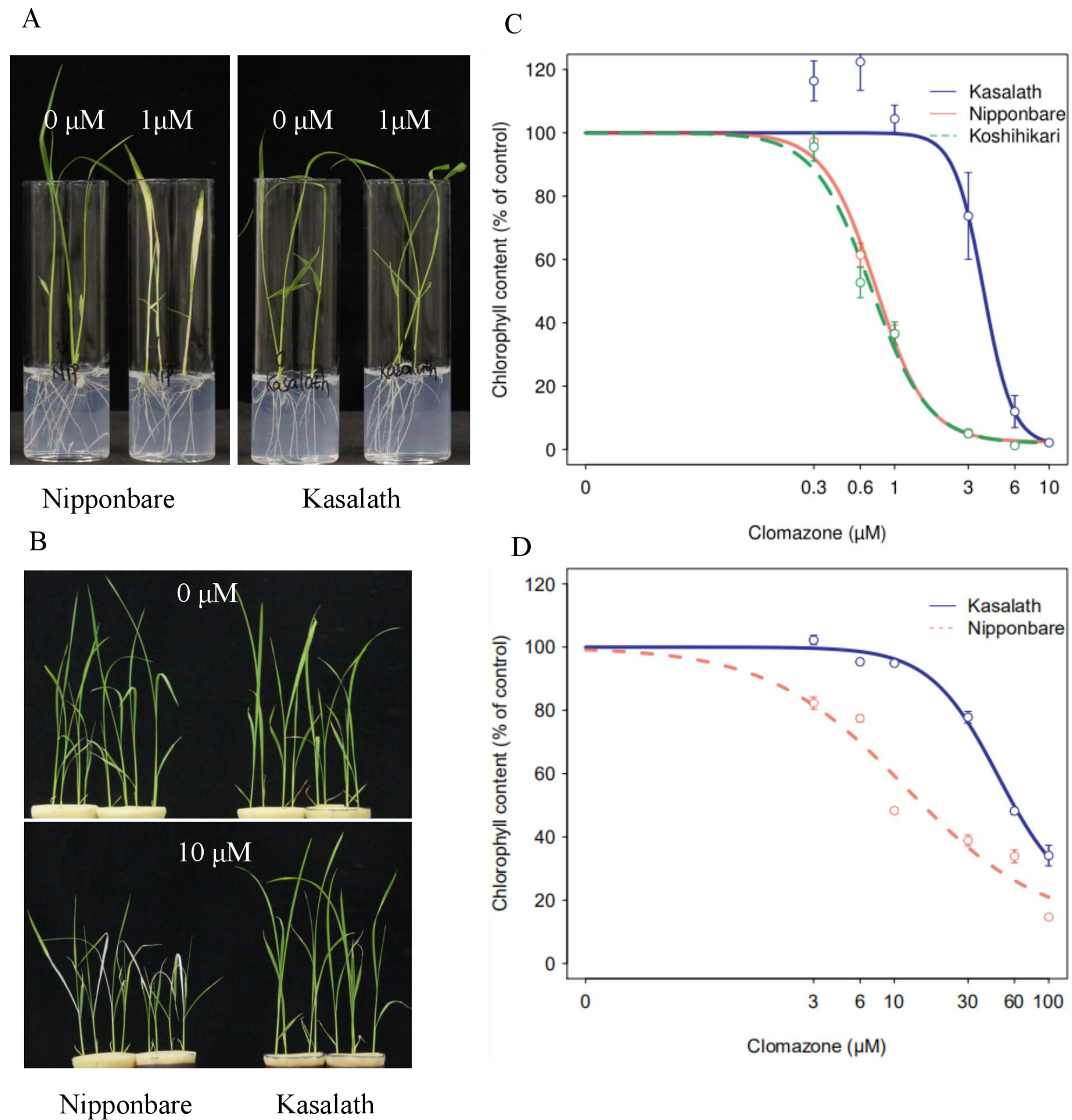


**Fig. 2.** Susceptibility to clomazone in different rice varieties. Susceptibility was evaluated by the relative chlorophyll content of seedlings. A, Seedlings of Nipponbare and Kasalath 6 d after clomazone application (1 µM). B, Seedlings of Nipponbare and Kasalath rice varieties 6 d after clomazone application (10 µM). C, Dose response curve of rice varieties to root-treated clomazone in the germinated seedlings. Bars represent standard deviation (n = 4). D, Dose response curve of rice varieties to root-treated clomazone in the 2-leaf stage seedlings. Bars represent standard deviation (n = 4).

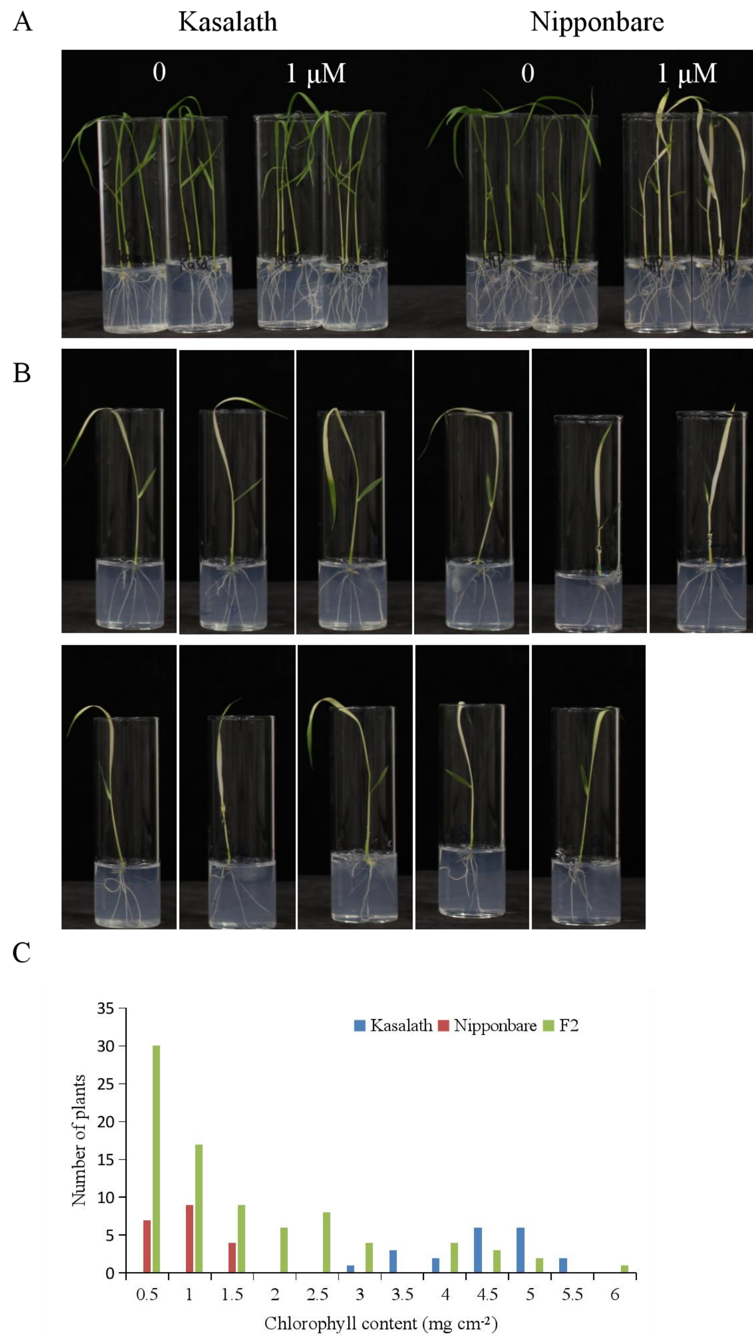


**Fig. 3.** Susceptibility to clomazone in Kasalath, Nipponbare and their progeny-F1 and F2 rice seedlings. Susceptibility was evaluated by the relative chlorophyll content of seedlings. A, Seedlings of Nipponbare and Kasalath rice varieties 6 d after clomazone application (1 μM). B, Seedlings of F1 derived from a cross between Kasalath and Nipponbare rice varieties 6 d after clomazone application (1 μM). The number of F1 plants is 11. C, Segregation of clomazone tolerance in an F2 population derived from a cross between Kasalath and Nipponbare rice varieties. The number of plants in each size range is shown (n=84 for the F2 population, n=20 for Kasalath and Nipponbare rice seedlings.

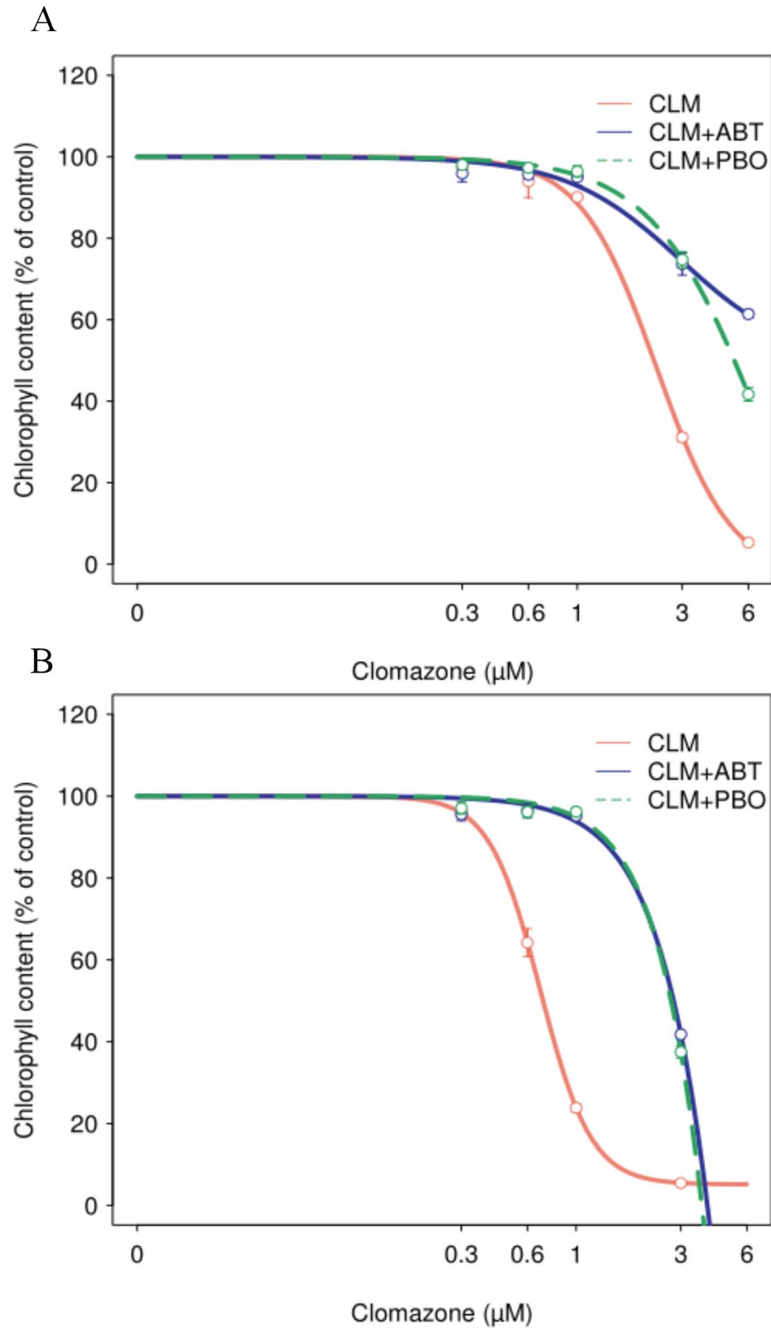


**Fig. 4.** Effects of P450 inhibitors-100 µM 1-aminobenzo-triazole (ABT) or 1 mM piperonyl butoxide (PBO) on susceptibility of Kasalath and Nipponbare rice varieties to clomazone (CLM). Bars represent standard deviation (n = 4). A, Dose response curve of Kasalath rice variety to clomazone with and without P450 inhibitors. B, Dose response curve of Nipponbare rice variety to clomazone with and without P450 inhibitors.

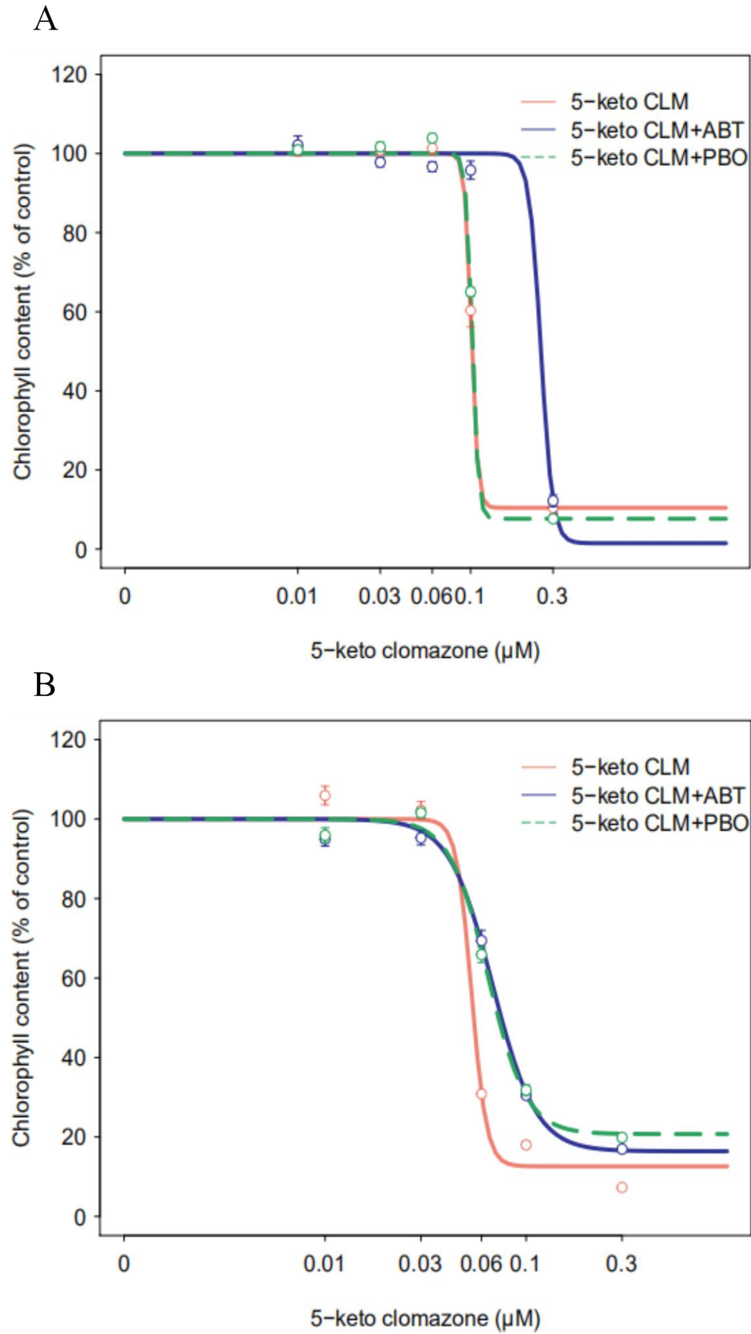


**Fig. 5.** Effects of P450 inhibitors-100 µM 1-aminobenzo-triazole (ABT) or 1 mM piperonyl butoxide (PBO) on susceptibility of Kasalath and Nipponbare rice varieties to 5-keto clomazone (5-keto CLM). Bars represent standard deviation (n = 4). A, Dose response curve of Kasalath rice variety to 5-keto clomazone with and without P450 inhibitors. B, Dose response curve of Nipponbare rice variety to 5-keto clomazone with and without P450 inhibitors.

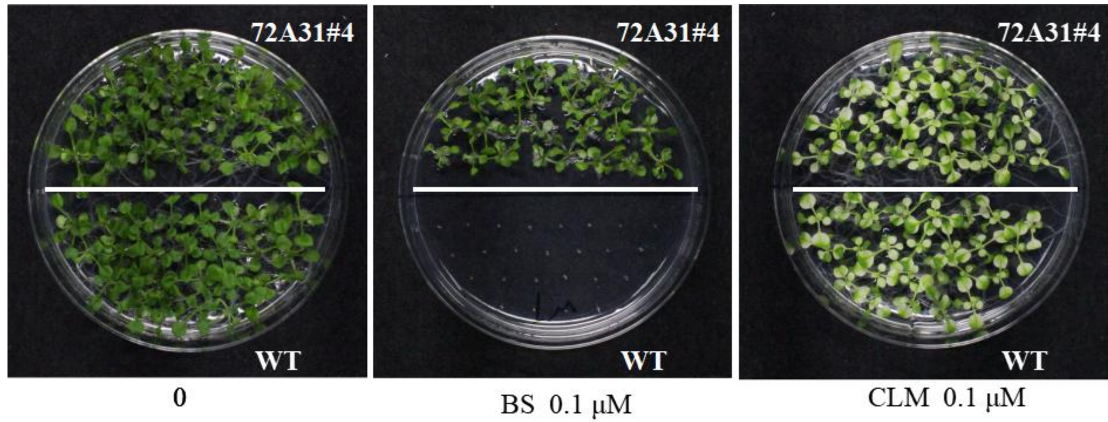


**Fig. 6.** Susceptibility to bispyribac sodium (BS) and clomazone (CLM) in Arabidopsis transformed with *CYP72A31*. WT, wild-type; 72A31#4-2, *CYP72A31* line; Seedlings grown for 14 d on Murashige and Skoog (MS) medium with 0.1 μM BS or 0.1 μM CLM.

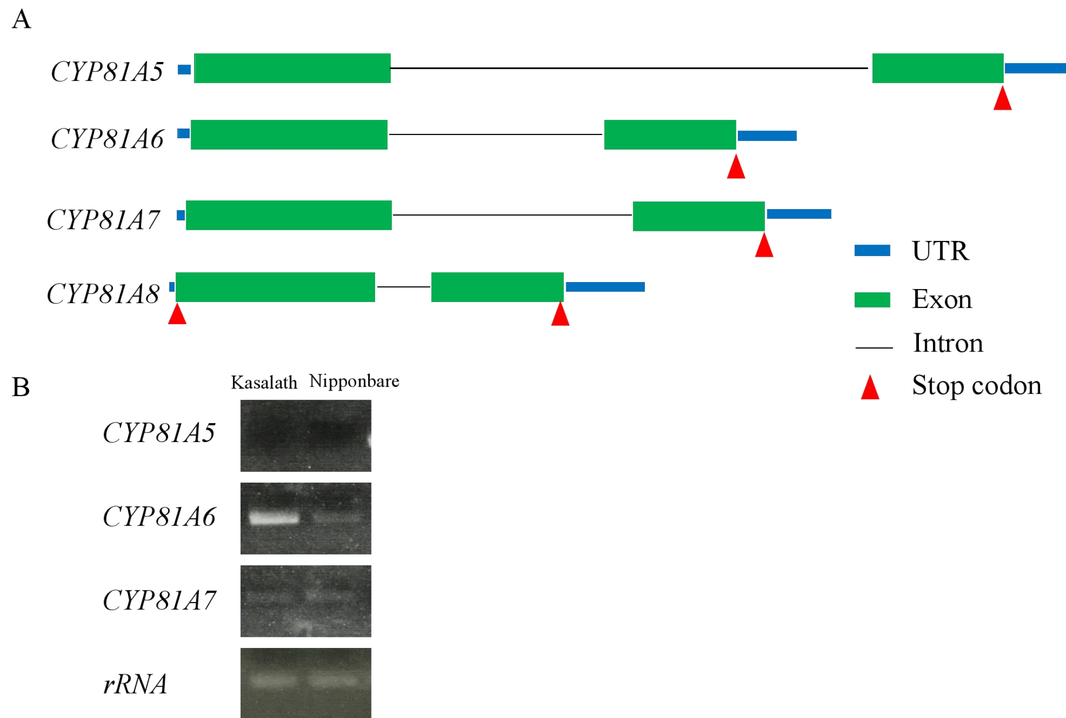


**Fig. 7.** Gene informations of *CYP81A*s of rice. A, The structures of *CYP81A*s. Blue column, untranslated region (UTR); Green column, exon; Black line, intron; Red triangle, stop codon. B, The expression level of *CYP81A*s in the shoot of Kasalath and Nipponbare rice seedlings; rRNA, internal control gene.

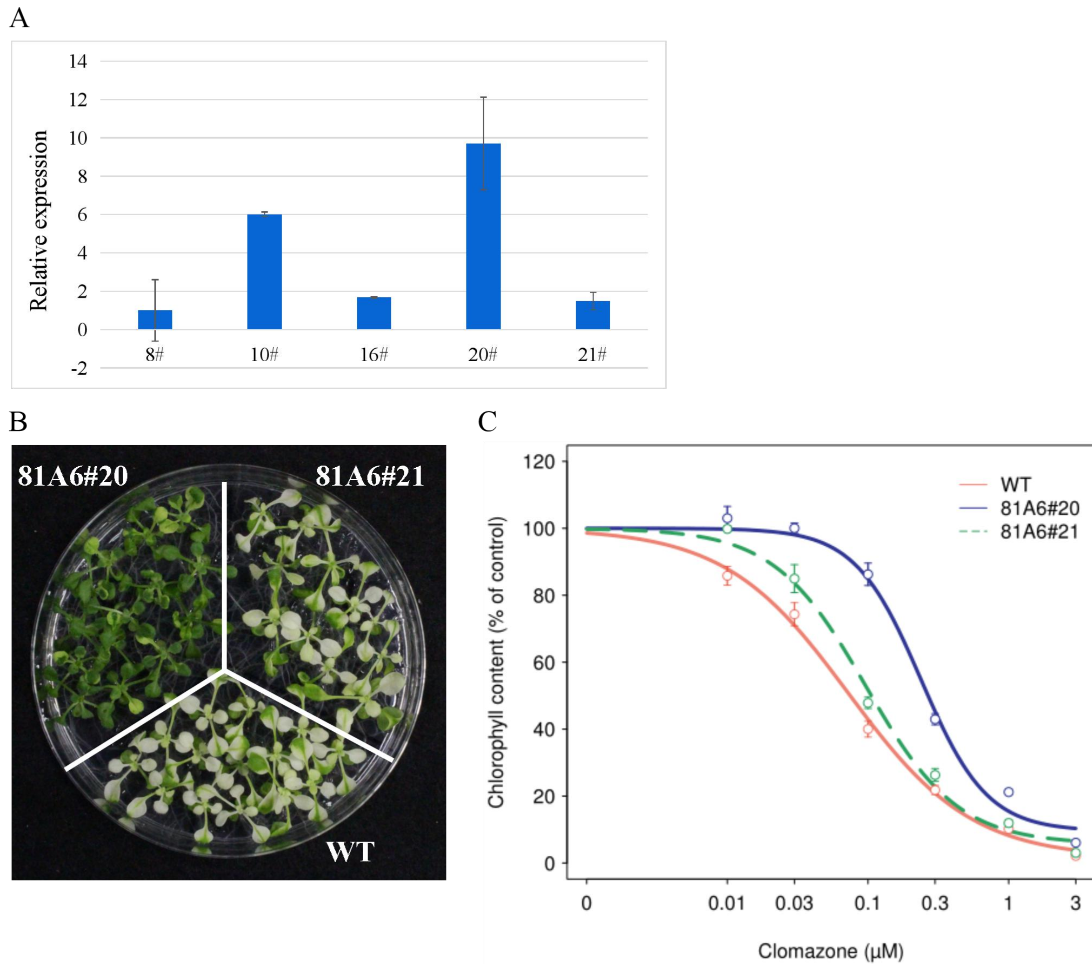


**Fig. 8.** Susceptibility to clomazone in Arabidopsis transformed with *CYP81A6*. WT, wild-type; 81A6#20-12 and 81A6#21-5,*CYP81A6* lines. A, Relative transcript level of *CYP81A6* of Kasalath rice variety in Arabidopsis transformed with *CYP81A6*. B, Seedlings grown for 14 d on Murashige and Skoog (MS) medium with 0.1 μM clomazone. C, Clomazone susceptibility of independent transgenic lines on MS medium containing clomazone. Susceptibility was evaluated by the relative chlorophyll content. Bars represent standard deviation (n = 4).

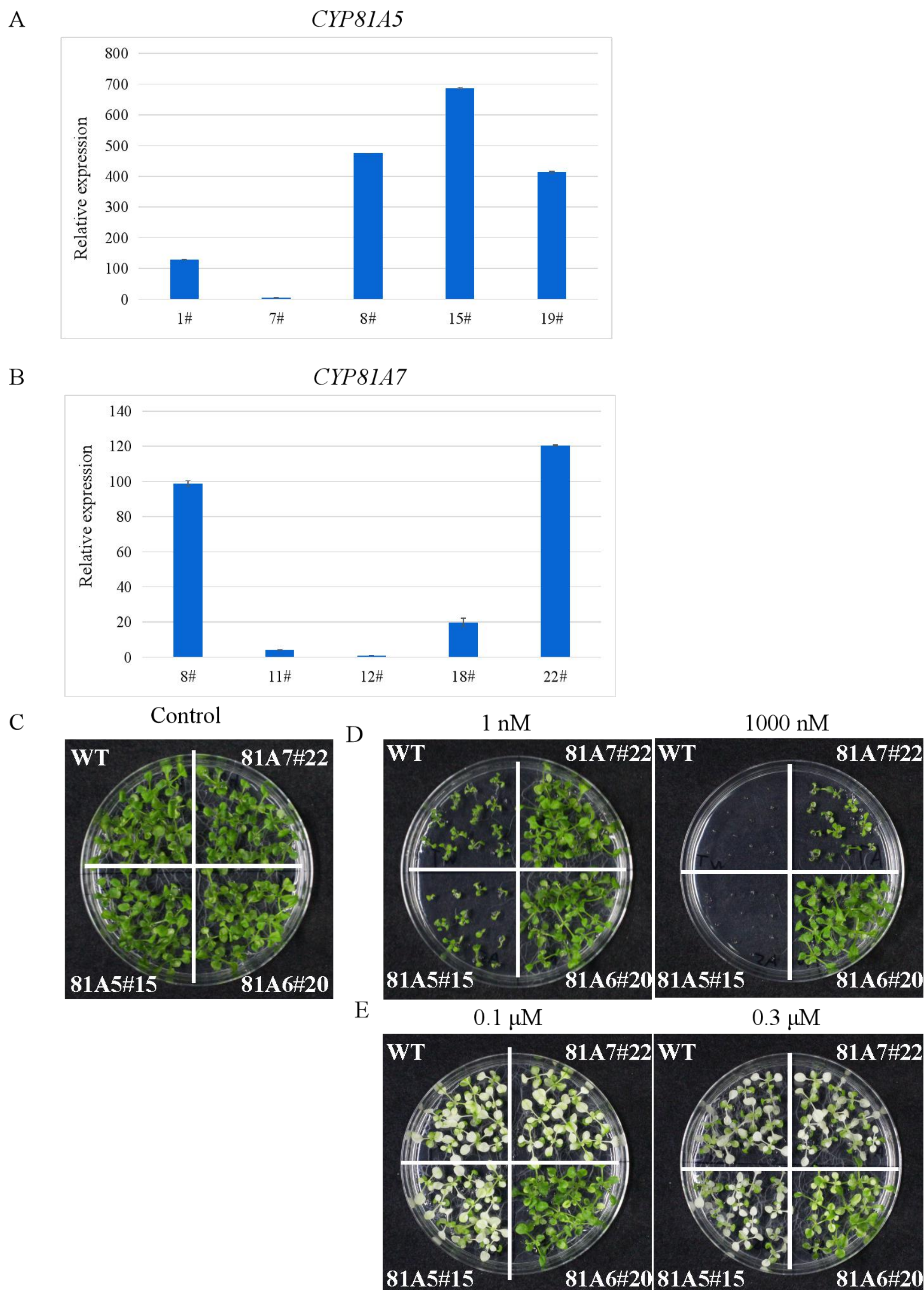


**Fig. 9.** Susceptibility to clomazone and bensulfuron-methyl (BSM) in Arabidopsis transformed with *CYP81A*s of Kasalath rice variety. Seedlings grown for 14 d on Murashige and Skoog (MS) medium with BSM (D) or clomazone (E). WT, wild-type; 81A5#15-3, *CYP81A5* line; 81A6#20-12, *CYP81A6* line; 81A7#22-1, *CYP81A7* line. A and B, Relative transcript level of *CYP81A5* and *CYP81A7* of Kasalath rice variety in Arabidopsis transformed with *CYP81A5* and *CYP81A7*. C, Control.

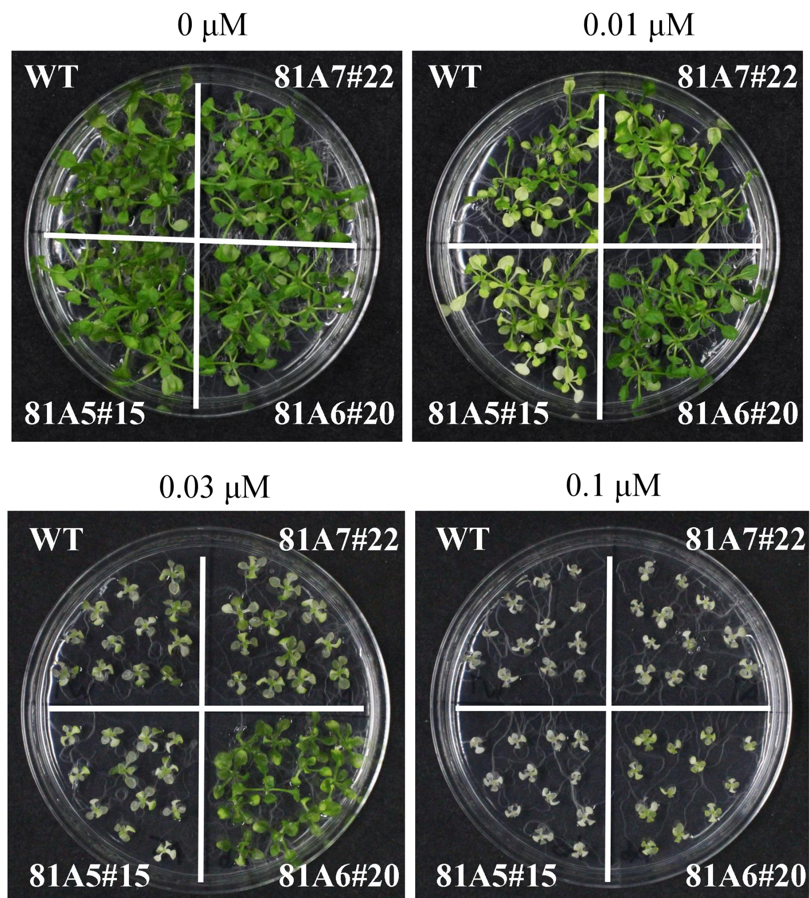


**Fig. 10.** Susceptibility to 5-keto clomazone in Arabidopsis transformed with *CYP81A*s of Kasalath rice variety. Seedlings grown for 14 d on Murashige and Skoog (MS) medium with 5-keto clomazone. WT, wild-type; 81A5#15-3, *CYP81A5* line; 81A6#20-12, *CYP81A6* line; 81A7#22-1, *CYP81A7* line.

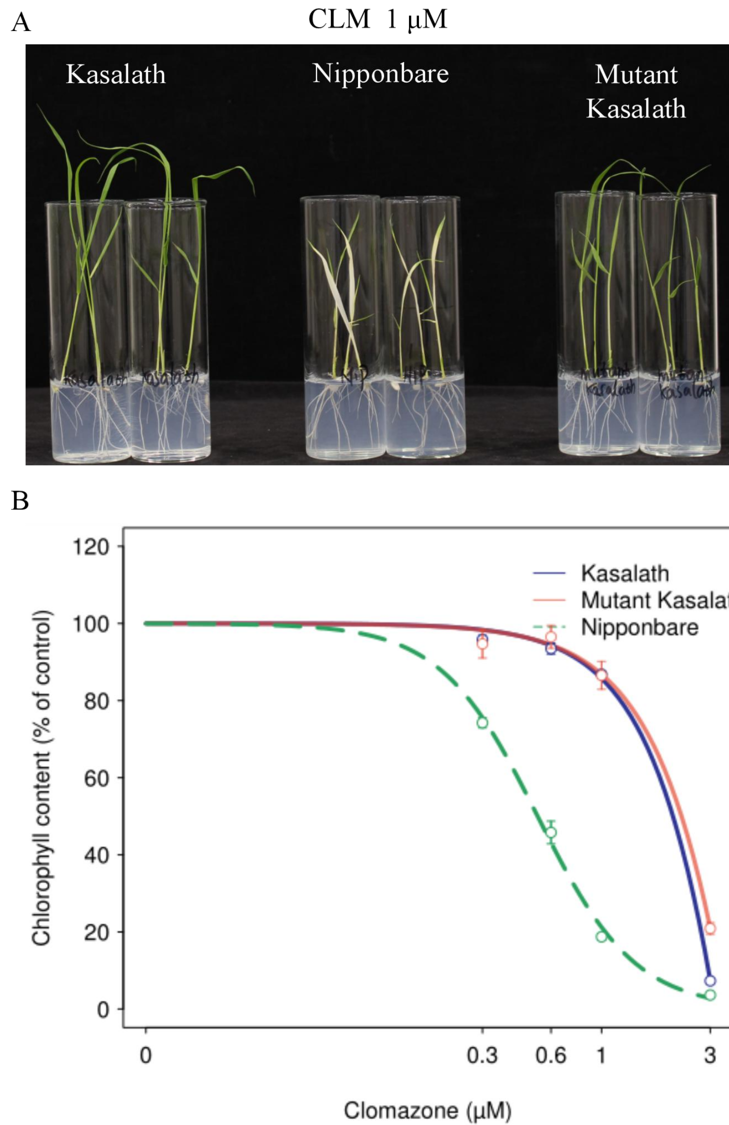


**Fig. 11.** Susceptibility to clomazone (CLM) in wild-type Kasalath, wild-type Nipponbare and mutant Kasalath rice seedlings. Susceptibility was evaluated by the relative chlorophyll content of seedlings. A, Seedlings of rice variety 6 d after clomazone application (1 µM). B, Dose response curve of rice varieties to root-treated clomazone in the germinated seedlings. Bars represent standard deviation (n = 4).

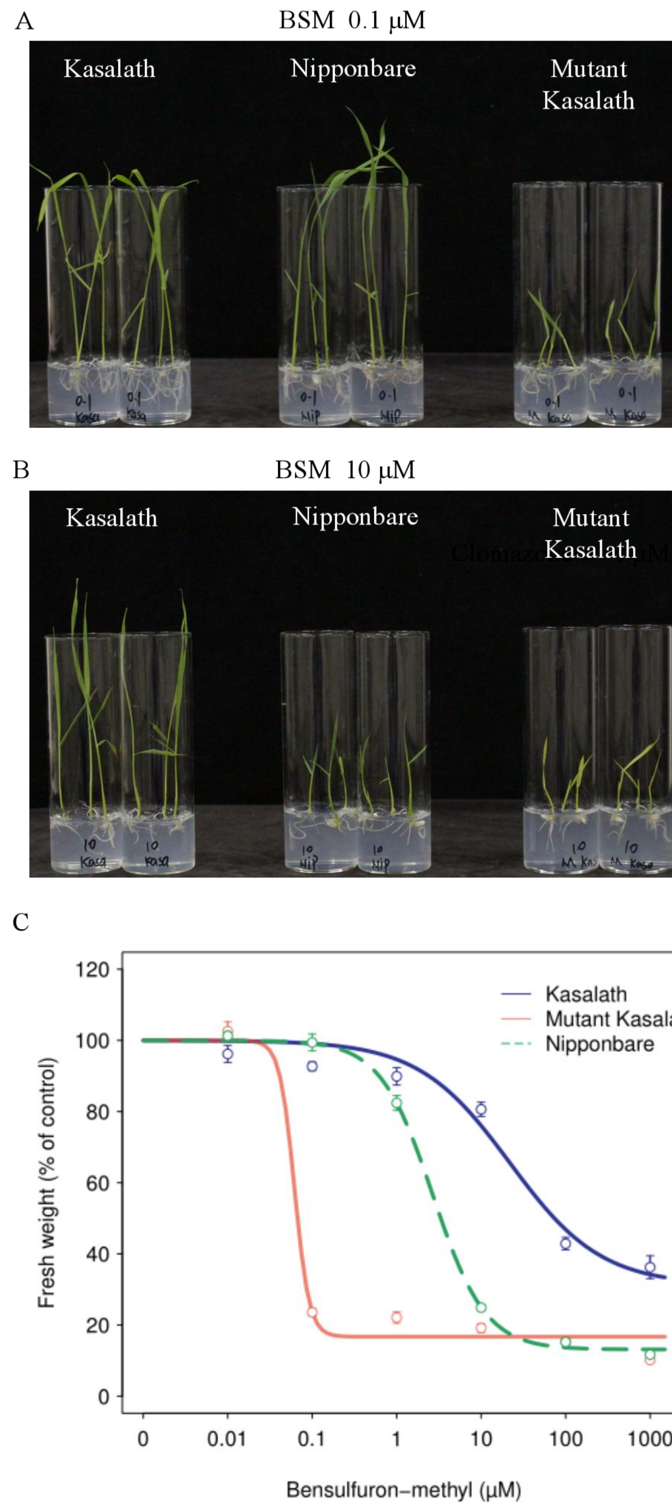


**Fig. 12.** Susceptibility to bensulfuron-methyl (BSM) in wild-type Kasalath, wild-type Nipponbare and mutant Kasalath rice seedlings. Susceptibility was evaluated by fresh weight of seedlings. A and B, Seedlings of rice variety 6 d after BSM application (0.1μM and 10 μM). C, Dose response curve of rice varieties to root-treated BSM in the germinated seedlings. Bars represent standard deviation (n = 4).

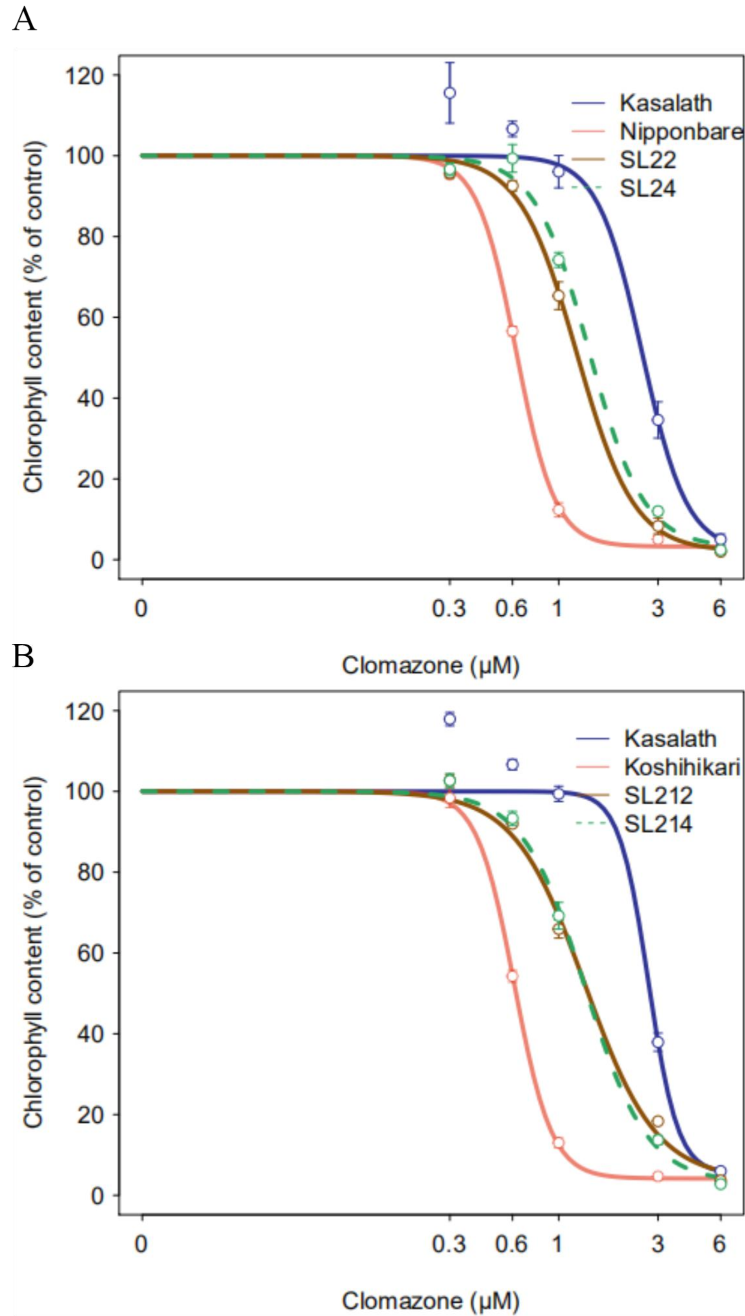


**Fig. 13.** Susceptibility of Kasalath, Nipponbare and CSSLs rice seedlings to clomazone. Susceptibility was evaluated by the relative chlorophyll content of seedlings. A, Dose response curve of rice varieties (Kasalath, Nipponbare, SL22 and SL24) to root-treated clomazone in the germinated seedlings. B, Dose response curve of rice varieties (Kasalath, Koshihikari, SL212 and SL214) to root-treated clomazone in the germinated seedlings. Bars represent standard deviation (n = 4).

# CHAPTER 2

## Role of CYP81A cytochrome P450s in clomazone metabolism in *Echinochloa phyllopogon*

**Abstract**

Herbicide-resistant *Echinochloa phyllopogon* has been found in the Sacramento Valley, California, USA. The resistant plants exhibit resistance to multiple herbicides with different modes of action, including acetolactate synthase (ALS) inhibitors and clomazone. Previously, Iwakami et al. (2014a, 2018) identified two cytochrome P450 monooxygenase (P450) genes, *CYP81A12* and *CYP81A21*, of which the overexpression is associated with resistance to ALS inhibitors and acetyl-coenzyme A carboxylase (ACCase) inhibitors. However, genes involved in clomazone resistance remain unknown, although a similar mechanism has been suggested as the basis of resistance. In this study, I revealed that clomazone resistance co-segregated with resistance to ALS inhibitors in $F_6$ progenies of crosses between susceptible and resistant *E. phyllopogon*. The two P450 genes conferred marked resistance to clomazone as well as to its active metabolite, 5-keto clomazone, in *Arabidopsis thaliana* (Arabidopsis), which was consistent with the reduced susceptibility to 5-keto clomazone observed in the resistant *E. phyllopogon*. The results suggest that overexpression of the two P450 genes are involved in the clomazone resistance of *E. phyllopogon*. The functional characterization of other *CYP81A*s in *E. phyllopogon* by ectopic expression in Arabidopsis showed that the additional two genes also metabolize clomazone and an ALS inhibitor. The four recombinant CYP81A proteins which were expressed in *E. coli*, could metabolize clomazone or 5-keto clomazone. Three of the four genes were upregulated by clomazone treatment in R populations. The results underpinned the importance of *CYP81A* subfamily of P450 in the herbicide metabolism of plants.

## 1. Introduction

The intensive application of herbicides has resulted in the rapid evolution of herbicide resistance in many weed species, which is now recognized as a threat to the global food security (Gould et al., 2018). Target site resistance (TSR) and non-target site resistance (NTSR) are the two primary mechanisms which are responsible for herbicide resistance (Yuan et al., 2007). TSR includes mutations that alter herbicide target sites such as amino acid substitutions or overproduction of target proteins (Powles and Yu, 2010). All the other mechanisms are grouped into NTSR, with enhanced herbicide metabolism as one of the most common NTSR mechanisms. Although the evolution of weed resistance, regardless of the mechanism, causes problems in agriculture, weeds with TSR are relatively easier to manage because (1) they can be controlled by herbicides with a different mode of action, and (2) resistance diagnosis for the choice of alternative herbicides can be performed by analyzing just one or a few genes encoding the herbicide target sites. However, weeds with NTSR, especially with metabolism-based resistance, are often more difficult to control, because they can potentially endow cross-resistance to chemically unrelated classes of herbicides, including yet-to-be-marketed herbicides, and thus, necessitate complex weed management strategies (Yu and Powles, 2014). Furthermore, our molecular understanding of NTSR is extremely limited, which hampers the rapid diagnosis of resistance and prescription of alternative herbicides.

Cytochrome P450 monooxygenase (P450) plays a key role in metabolism-based resistance (Yuan et al., 2007). P450s are the heme-thiolate enzymes that typically catalyze monooxygenation or hydroxylation reactions depending on NADPH-P450 oxidoreductases that catalyze the transfer of electrons from NADPH to P450s (Bak et al., 2011). Plants contain hundreds of P450 genes, some of which are known to metabolize herbicides. A slight modification of the chemical structure of herbicides by a P450 reaction often results in the inactivation of herbicides (Siminszky, 2006). The involvement of P450s in herbicide resistance has been recognized in multiple weed species (Christopher et al., 1991; Maneechote et al., 1997; Ma et al., 2013). Despite the importance of P450s in plant herbicide response, the existence of many genes in plant genomes has hindered the molecular identification of genes involved in herbicide resistance.

*Echinochloa phyllopogon* (Stapf.) Koss., a predominantly self-pollinated allotetraploid plant, is a major weed in the rice fields of California, USA. The continuous application of thiocarbamate herbicides for more than 20 years and of fenoxaprop-ethyl for a few years led to the resistance of *E. phyllopogon* in at least nine herbicides from different chemical groups (Fischer et al., 2000; Osuna et al., 2002; Ruiz-Santaella et al., 2006; Bakkali et al., 2007; Yasuor et al., 2008, 2009, 2012). Previous studies revealed that the resistance to most herbicides such as acetolactate synthase (ALS) inhibitors, acetyl-coenzyme A carboxylase (ACCase) inhibitors and clomazone is mainly caused by enhanced herbicide metabolism (Ruiz-Santaella et al., 2006; Bakkali et al., 2007; Yasuor et al., 2009, 2010). Additionally, two *CYP81A* P450 genes, *CYP81A12* and *CYP81A21*, were identified to play a key role in the resistance to two chemical classes of ALS inhibitors, bensulfuron-methyl (BSM) and penoxsulam and three chemical classes of ACCase inhibitors in *E. phyllopogon* (Iwakami et al., 2014a, 2018). However, the resistance mechanisms to other herbicides remain unknown.

The herbicide clomazone has been used in numerous agricultural crops for its excellent activity on weeds (Yasuor et al., 2008; Schreiber et al., 2015). Clomazone is taken up by the roots and emerging shoots and is carried into the xylem to plant leaves (Senseman, 2007). Once absorbed by the plants, clomazone is converted to an active form, 5-keto clomazone, which inhibits the deoxyxylulose 5-phosphate synthase in the first committed step of non-mevalonate pathway, synthesizing isoprenoid pyrophosphate and dimethylallyl diphosphate, the precursors of isoprenoid molecules such as chlorophylls, carotenoids, and tocopherols (Ferhatoglu and Barrett, 2006). Consequently, plants treated with clomazone are bleached (white, yellow, or pale-green appearance) (Duke et al., 1991). Having a unique mode of action, clomazone is an excellent choice for herbicide resistance management. However, the multiple herbicide-resistant *E. phyllopogon* exhibits resistance to clomazone, despite the lack of application history in the rice fields of California. Previous studies on the mechanism of clomazone resistance in *E. phyllopogon* showed that clomazone is rapidly metabolized into oxidized forms, suggesting the involvement of P450s in resistance metabolism (Yasuor et al., 2008, 2010). Although P450s have been

suggested to play an important role in clomazone metabolism, the corresponding genes have not been yet identified.

In this chapter, I investigated the mechanism of clomazone resistance in *E. phyllopogon* and compared it with that of ALS inhibitors. My experiments using $F_6$ progenies of crosses between resistant and susceptible *E. phyllopogon* and *Arabidopsis thaliana* (Arabidopsis) transformed with *CYP81A12* or *CYP81A21* suggested that clomazone resistance results from the overexpression of *CYP81A12* and *CYP81A21* as in the case of resistance to ALS inhibitors. Further functional studies of other *CYP81A*s in *E. phyllopogon* suggested that some of them have clomazone and BSM metabolizing function, highlighting the important roles of the *CYP81A* subfamily in the herbicide metabolism of plants.

## 2. Materials and methods

### *2.1 Plant materials*

In this study, I used the resistant line 511 and susceptible line 401 of *E. phyllopogon* originated from rice fields in the Sacramento Valley of California in 1997 (Tsuji et al., 2003) and self-pollinated for three successive generations in Japan; 50 $F_6$ progenies of crosses between susceptible and resistant *E. phyllopogon* that were previously evaluated for BSM susceptibility (Iwakami et al., 2014a); Arabidopsis (Col-0) transformed with the resistant-line alleles *CYP81A12*, *CYP81A21*, or *CYP81A22* (Iwakami et al., 2014b); and four $T_3$ lines of *CYP81A12* and *CYP81A21* (81A12#19-2, 81A12#21-10, 81A21#6-1, and 81A21#9-1) that were found to have the highest and second highest expression of *CYP81A12* and *CYP81A21* out of five and four of total lines, respectively.

### *2.2 Clomazone and 5-keto clomazone susceptibility of E. phyllopogon*

The seeds of *E. phyllopogon* were washed with 70% (v/v) ethanol for 30 s, sterilized twice with 2.5% (w/v) and 0.2% (w/v) sodium hypochlorite, containing 0.1% (v/v) Tween 20, for 15 min, respectively, and washed three times in sterile water. The seeds were plated on moist filter paper in petri dishes and cultured in a growth chamber at 25°C under fluorescent light (approximately 300 μmol $m^{-2}$ $s^{-1}$) with a 12-h photoperiod (d 0). The germinated seeds were transferred to glass tubes which contain murashige and skoog (MS) medium (Murashige and Skoog, 1962) supplemented with clomazone (0, 0.3, 0.6, 1, 3, 6 or 10 μM) at the $4^{th}$ day. After 6 d, the shoots were excised and soaked in 100% (v/v) dimethyl sulfoxide (DMSO) (50 ml/g FW) in a glass tube (Barnes et al., 1992; Hiscox and Israelstam, 1979; Monje and Bugbee, 1992). The tube was incubated at 25°C in the dark for 2 d. The concentration of extracted chlorophyll (four replicates) was calculated based on the absorbance value at 645 nm and 663 nm as described previously (Arnon, 1949). The absorbance was measured using Multiskan GO (Thermo-Fisher Scientific, Waltham, MA, USA). $I_{50}$ was estimated by non-linear regression using the log-logistic model with the 'drc' package (Ritz et al., 2015) in R 3.3.3 (Team, 2016). The treatment of herbicides were replicated 4 times and the experiment was performed at least twice.

For root treatment at 2-leaf stage, the seeds of *E. phyllopogon* were incubated in distilled water at 25°C in the dark for 2 d. The seeds were transferred to a growth chamber at 25°C under fluorescent light (approximately 300 μmol $m^{-2}$ $s^{-1}$) with a 12-h photoperiod (d 0). Plants were transferred to 250 ml vessels, containing 170 ml of 1/10 concentration of Kasugai nutrient solution (Ohta, 1970) at d 8 and of full concentration at d 11. The solution was replaced every three days. The roots of 2-leaf stage plants were dipped into clomazone solution (0, 10, 30, 60, 100 or 300 μM) for 24 h. At 6 d after the application, the shoots were excised, and soaked in 100% (v/v) DMSO (50 ml/g FW) in a glass tube (Barnes et al., 1992; Hiscox and Israelstam, 1979; Monje and Bugbee, 1992). The chlorophyll content (four replicates) was measured as described above. The treatment of herbicide was replicated 4 times and the experiment was performed at least twice.

For leaf treatment at 2-leaf stage, the seeds of the susceptible and resistant lines were incubated in distilled water at 25°C in the dark for 2 d. The seeds were transferred to a growth chamber at 25°C under fluorescent light (approximately 300 μmol $m^{-2}$ $s^{-1}$) with a 12-h photoperiod (d 0). Plants were transferred to 250 ml vessels, containing 170 ml of 1/10 concentration of Kasugai nutrient solution (Ohta, 1970) at d 8 and of full concentration at d 11. The solution was replaced every three days. The shoots of 2-leaf stage plants were dipped into clomazone solution (0, 10, 30, 60, 100 or 300 μM) or 5-keto clomazone solution (0, 1, 3, 6, 10, 30, 60 or 100 μM) for 4 h. At 6 d after the application, the shoots were excised, and weighed the weight firstly, then soaked in 100% (v/v) DMSO (50 ml/g FW) in a glass tube (Barnes et al., 1992; Hiscox and Israelstam, 1979; Monje and Bugbee, 1992). The chlorophyll content (four replicates) was measured as described above. The treatments of herbicides were replicated 4 times and the experiment was performed at least twice.

The cultivation of $F_6$ lines and clomazone application were conducted as described above-leaf treatment with the following modifications: 100 μM of clomazone was applied; clomazone susceptibility of each line was evaluated visually.

*2.3 Arabidopsis transformation*

Vector construction for *CYP81A14*, *CYP81A15*, *CYP81A18*, *CYP81A23*, *CYP81A24*, and *CYP81A26* was performed as described previously with some modifications (Iwakami et al., 2014a). Briefly, the full-length cDNA sequences of *CYP81A23* and *CYP81A26* were amplified by PCR using cDNA prepared from the shoot of 3-leaf stage resistant line with the primers listed in Table 2. The full-length sequences of *CYP81A14*, *CYP81A15*, *CYP81A18*, and *CYP81A24* were amplified by PCR using cDNA prepared from bispyribac-sodium (BS) treated plants since the transcription of the four genes is induced by the BS application (Iwakami et al., 2014b). The amplicons were sub-cloned into pENTR-D-TOPO (Thermo-Fisher Scientific). The binary vectors were produced with GATEWAY LR clonase II (Thermo-Fisher Scientific) from the sub-clones and pB2GW7 (Karimi et al., 2002). The binary vectors were transformed into *Agrobacterium tumefaciens* EHA105 as described previously (Hofgen and Willmitzer, 1988). The transformed *A. tumefaciens* was used for Arabidopsis (Col-0) transformation by the floral dip method (Clough and Bent, 1998). $T_3$ homozygous lines with a single copy of transgene were selected based on the segregation ratio against bialaphos at $T_2$ and $T_3$.

For transformation of mutant *CYP81A12*, *CYP81A15* and *CYP81A21*, site-directed mutagenesis was conducted firstly. After checking the sequence to confirm the successful mutant, the mutant genes were transferred into pB2GW7 vector using GATEWAY LR clonase II (Thermo-Fisher Scientific). The subsequent process were same as described above.

*2.4 Real-time PCR for newly established transgenic Arabidopsis lines*

$T_3$ homozygous Arabidopsis lines were grown on MS (Murashige and Skoog, 1962) medium for 10-14 d. RNA was extracted from 4-5 plants using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using ReverTra Ace (Toyobo, Osaka, Japan). Real-time PCR was conducted with SYBR Green Realtime PCR Master Mix (Toyobo) on the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the primers listed in Table 2. Data were normalized with *GAPDH* (M17851) using the ΔΔCT method (Schmittgen and Livak, 2008). The RNA extraction for every line was replicated 3 times.

*2.5 Herbicide susceptibility of transgenic Arabidopsis*

The seeds of transgenic Arabidopsis were washed with 70% (v/v) ethanol for 30 s, sterilized with 0.1% (w/v) sodium hypochlorite, containing 0.1% (v/v) Tween 20, for 3 min, and washed three times in sterile water. The sterilized seeds were placed on solid MS medium (Murashige and Skoog, 1962) in petri dishes supplemented with clomazone (0, 0.03, 0.1, 0.3, 1, 3, 10 or 30 μM), 5-keto clomazone (0, 0.01, 0.03, 0.1, 0.3, 1 or 3 μM) or BSM (1, 3, 10, 30, 100, 300 or 1000 nM). The dishes were kept in a growth chamber at 22°C under fluorescent light (approximately 70 μmol $m^{-2}$ $s^{-1}$) and a 12-h photoperiod for 14 d. The position of each dish was changed every 1-2 d. The chlorophyll content of 10 seedlings (four replicates) was measured at 14 d after the treatment as described above. The treatments of herbicides were replicated 4 times and the experiment was performed at least twice.

*2.6 Real-time PCR for comparison of the expression level of CYP81As in E. phyllopogon between treated and non-treated clomazone*

The seeds of the susceptible and resistant lines were incubated in distilled water at 25°C in the dark for 2 d. The seeds were transferred to a growth chamber at 25°C under fluorescent light (approximately 300 μmol $m^{-2}$ $s^{-1}$) with a 12-h photoperiod (d 0). Plants were transferred to 250-ml vessels, containing 170 ml of 1/10 concentration of Kasugai nutrient solution (Ohta, 1970) at d 8 and of full concentration at d 11. The solution was replaced every three days. The shoot of 2-leaf stage plants was dipped into clomazone solution (100 μM) for 4 h at d 11. Before clomazone application and after clomazone application 0 h, 4 h, 8 h, 12 h and 24 h, the shoot samples were collected and quick-frozen in liquid nitrogen. Samples were ground to powder using pre-refrigerant mortar and pestle, and total RNA was extracted from 4-5 plants using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). qRT-PCR was performed as described in "2.4". The RNA extraction for every line was replicated 3 times.

*2.7 Escherichia coli transformation and expression of the P450s*

The coding sequences of *CYP81A*s and *ATR2* without N-terminal transmembrane regions were amplified by PCR using the PrimeSTAR MAX DNA polymerase or the Tks Gflex DNA polymerase (Takara) and appropriate primer sets (Table 2). N-terminal transmembrane regions of *CYP81A*s were predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The amplified fragments of *CYP81A*s and *ATR2* were inserted into the NcoI-HindIII site of the pET28a-(+) vector (Merck Millipore, Billerica, MA, USA) and the pCDF-1b vector (Merck Millipore), respectively. The inserted DNA sequences were confirmed by Sanger sequencing. N-terminal amino acid sequences of CYP81As were replaced to that of CYP2C3 by inverse PCR method using the pET28 vectors carrying CYP81As as template DNA. The resultant plasmid names were summarized in Table 3.

The gene encoding 5-aminolevurinic acid synthase (HemA) expression plasmid was constructed as follows. The gene was synthesized and cloned into the NcoI-HindIII site of pCDF-1b. After confirmation of the inserted DNA sequences, hemA with T7 promoter and terminator region was amplified and cloned into the Bsu36I site of pCDF-1b carrying ATR2 (pCDF-ATR2) using the NEBuilder HiFi DNA Assembly Master Mix. The resultant plasmid was named pCDF-ATR2-hemA.

Each pET28 vector carrying *CYP81A*, pCDF-ATR2-hemA, and pGro7 (Takara) encoding chaperonin GroEL/GroES were used to transform *Escherichia coli* (*E. coli*) C41(DE3) (Lucigen, Middleton, WI, USA). The transformants were cultured on the basis of previously described auto-induction method (Miki and Asano, 2014). In brief, each colony of transformant was inoculated into LB containing glucose (1% w/v), kanamycin (50 μg/mL), streptomycin (50 μg/mL), and chloramphenicol (34 μg/mL), and cultured at 37°C for overnight. Ten micro liter of culture was transferred to 1 mL of an auto-induction medium. After the culture at 26°C for 24 h, herbicide (0.2 mM) and arabinose (2 mg/mL) was added and further cultured at 30°C for 24 h.

Samples were analyzed using an LC–MS/MS system equipped with a COSMOSIL 2.5C18-MS-II column (75 mm × 2.0 mm i.d.; Nacalai Tesuque, Kyoto, Japan) under the following condition; mobile phase A, 0.1% formic acid in water, mobile phase B, acetonitrile, and 10–60% linear gradient of B for 8 min and 60% B for 1 min delivered at 0.4 mL/min. MS was simultaneously performed in positive- or

negative- ion mode using a LCMS-8030 (Shimadzu). Multi reaction monitoring was used to detect putative hydroxylated products of herbicides.

## 3. Results

### *3.1 Clomazone and 5-keto clomazone susceptibility of E. phyllopogon*

Dose response of R line and S line of *E. phyllopogon* were conducted using root treatment at different leaf stages (Fig. 1A & B), and the resistant index is more than 2 which is consistent with previous report that followed the conventional into-the-water clomazone treatment in greenhouse (Yasuor et al., 2008).

The susceptible line of *E. phyllopogon* was more affected by the clomazone application compared to the resistant line using leaf treatment. The most prominent difference between the susceptible and resistant lines was observed in the leaf color, although a significant difference was also observed in the shoot fresh weight (Fig. 1C & D). The bleaching symptom was limited to the emerging leaves, especially, the third leaf (Fig. 1F), resulting in a 60% higher chlorophyll content in the whole shoot of treated plants compared with the non-treated plants. Thus, instead of evaluating the whole shoots, I focused on the chlorophyll content of the third leaf.

The chlorophyll content in the third leaf of susceptible plants decreased with the increasing clomazone concentration of 30 μM or higher (Fig. 1C). The 100-μM clomazone application resulted in the total bleaching of the third leaf of susceptible lines, but not of that of resistant line (Fig. 1F). The $I_{50}$ of susceptible and resistant lines were 62.6 μM and 582 μM, respectively, resulting in an $I_{50}$ ratio (R/S) of 9.3. A similar $I_{50}$ ratio (R/S) of 6.9 was obtained from the 5-keto clomazone application, although the $I_{50}$ of susceptible (10.8 μM) and resistant (74.3 μM) lines were lower than those obtained from the clomazone application (Fig. 1E).

### *3.2 Segregation of resistance to clomazone and BSM in $F_6$ lines*

To investigate whether the resistance mechanism to clomazone and ALS inhibitors is identical, 50 $F_6$ progenies of crosses between resistant and susceptible plants were studied. Previously, the susceptibility of $F_6$ lines to BSM-an ALS inhibitor, were evaluated: 19 lines were resistant, 30 lines were susceptible, and one line was a segregate (Iwakami et al., 2014a; Table 1). In the 50 F6 lines, high

transcript levels of *CYP81A12* and *CYP81A21* perfectly co-segregated with BSM resistance. In this study, the susceptibility of each line to clomazone was evaluated by the color of the third leaf. Among the 50 $F_6$ lines, 19 lines were resistant to clomazone (green third leaf), 30 lines were susceptible (bleached third leaf), whereas one line was a segregate (Table 1). Clomazone resistance also co-segregated with high transcript levels of *CYP81A12* and *CYP81A21*, suggesting an identical resistance mechanism to BSM and clomazone (Table 1).

### *3.3 Clomazone susceptibility of Arabidopsis transformed with CYP81A12 or CYP81A21*

Next, I investigated the clomazone susceptibility of Arabidopsis transformed with *CYP81A12* or *CYP81A21*, the P450 genes that are overexpressed in resistant *E. phyllopogon*. The Arabidopsis lines possess a single copy of transgene and exhibit significant resistance level to ALS inhibitors (Iwakami et al., 2014a). The 0.1 μM clomazone application resulted in the almost total bleaching of the true leaves of wild-type Arabidopsis, but not that of *CYP81A12*- and *CYP81A21*-transformed lines (Fig. 2A & B). The $I_{50}$ ratio of transgenic and wild-type lines was 6.3, 6.7, 6.8, and 12.9 for 81A12#19-2, 81A21#9-1, 81A12#21-10, and 81A21#6-1, respectively. Thus, the susceptibility to clomazone was positively correlated with the transgene expression level (Fig. 2C & D). The results suggest that the two P450 genes play a major role in clomazone resistance in *E. phyllopogon*.

### *3.4 Herbicide susceptibility of Arabidopsis transformed with CYP81As*

The results of transformed Arabidopsis with *CYP81A12* or *CYP81A21* suggested that *CYP81A* P450s metabolize not only ALS inhibitors but also clomazone. The similar expression levels of *CYP81As*, except for *CYP81A22*, between the susceptible and resistant lines imply that the genes are not likely be involved in the enhanced clomazone metabolism of the resistant line. *E. phyllopogon* possesses at least 12 *CYP81A*s, including *CYP81A12* and *CYP81A21*, three of which are frameshift-type pseudogenes (Iwakami et al., 2014a). Here, I compared the susceptibility of Arabidopsis transformed with different *CYP81A*s (except for the three pseudogenes) to BSM and clomazone. Lines with the highest expression level of the transgenes

among at least three independently transformed lines were selected for this study (Table 4). The 1 nM BSM application resulted in the growth inhibition of wild-type Arabidopsis, whereas the 3 nM BSM application in the plant death (Fig. 3A & B). Arabidopsis transformed with *CYP81A12* or *CYP81A21* showed resistance to BSM application up to 1,000 nM. Thus, the resistant index was more than 1,000 as previously reported (Iwakami et al., 2014a). Arabidopsis transformed with *CYP81A15*, *CYP81A18*, or *CYP81A24* also exhibited decreased susceptibility to BSM, but the resistance level was lower than that of those transformed with *CYP81A12* or *CYP81A21* (Fig. 3B). The resistance index of Arabidopsis transformed with *CYP81A15*, *CYP81A18*, or *CYP81A24* were 10-fold, 10-fold, and 100-fold higher than that of wild-type Arabidopsis, respectively.

All the BSM resistant lines, except for the *CYP81A18* line, were less damaged in the presence of clomazone than wild-type Arabidopsis (Fig. 3C). Arabidopsis transformed with *CYP81A15* exhibited the highest resistance level to clomazone, whereas those transformed with *CYP81A12* or *CYP81A21* showed the highest resistance to BSM (Fig. 3B & C).

*3.5 Susceptibility of Arabidopsis transformed with mutant CYP81As to clomazone*

*CYP81A15* which confers much higher level resistance to clomazone in Arabidopsis than other *CYP81A*s, is an interesting topic, urging me to find the reason. I compared the residue of amino acid sequences of CYP81As of both R line and S line of *E. phyllopogon* and found that there is a polymorphism between CYP81A15 and other CYP81As at site 122-other CYP81As is Leucine while that of CYP81A15 is replaced by proline (Fig. 4). It is known that a single amino acid substitution can cause altered regiospecificity and catalytic efficiency in P450s, especially polumorphisms in SRSs (Kahn et al., 2001; Schalk and Croteau, 2000). Polymorphisms at substrate recognition sites (SRSs) in P450s can influence their metabolic functions. There are 6 putative SRSs in P450s which are proposed to play important roles in the enzyme activities (Gotoh, 1992; Iwakami et al., 2014a). Therefore, the involvement of the polymorphism in CYP81A15 (L122P) in clomazone resistance should be investigated, especially the polymorphysm is in SRS.

Vectors pENTR-D-TOPO carrying *CYP81A12*, *CYP81A15* and *CYP81A21* were used for site-directed mutagenesis to generate *CYP81A*s mutant plasmids. The plasmids were transferred into pB2GW7 vector using GATEWAY system (LR reaction) and then Arabidopsis transformation were performed. After selected T3 homozygous lines, the lines which have similar transcript level between wild type and mutant type Arabidopsis expressing *CYP81A12*, *CYP81A15* and *CYP81A21* were chose to conduct susceptibility test to both BSM and clomazone (Fig. 5).

The results showed that mutant type and wild type Arabidopsis carrying *CYP81A15* had similar response to BSM and clomazone. However, mutant type Arabidopsis carrying *CYP81A12* and *CYP81A21* had increased susceptibility to both BSM and clomazone compared with wild type ones (Fig. 6). These results suggested that the polymorphism in *CYP81A15* is not the vital SRS for its high-level clomazone resistance, but this SRS is very important to *CYP81A12* and *CYP81A21* for metabolizing both BSM and clomazone.

*3.6 Susceptibility of Arabidopsis transformed with CYP81As to 5-keto clomazone*

Next, I investigated the susceptibility of the four clomazone-resistant Arabidopsis lines (*CYP81A12*, *CYP81A15*, *CYP81A21*, and *CYP81A24*) to 5-keto clomazone, an active form of clomazone. The 0.03 μM 5-keto clomazone application resulted in the total bleaching of wild-type Arabidopsis but did not affect the growth of Arabidopsis transformed with *CYP81A12*, *CYP81A15*, *CYP81A21*, or *CYP81A24* (Fig. 7). The transgenic lines, except for *CYP81A15*, were severely affected at 1 μM 5-keto clomazone application. Consistent with clomazone application, Arabidopsis transformed with *CYP81A15* exhibited the highest resistance level.

*3.7 Transcript levels of CYP81A genes in R and S E. phyllopogon between treated and non-treated clomazone*

Among the four genes, *CYP81A12* and *CYP81A21* transcripts were particularly abundant in the shoot of R line seedlings at 2-leaf stage before clomazone treatment, which are consistent with previous report (Iwakami et al., 2014a). The expression of *CYP81A12*, *CYP81A21* and *CYP81A24* were induced by clomazone application in R

line seedlings, even though that of S line also increased, the difference is not significant statistically (Fig. 8). For *CYP81A12*, the expression level were higher significantly after clomazone treatment 0 h, 4 h and 8 h, while that of *CYP81A21* was still kept in a high horizon after clomazone application 12 h, compared with control in R plants. Moreover, transcript level of *CYP81A21* of R line were induced by clomazone remarkably higher than control after clomazone treatment 0 h, 4 h and 8 h compared with control. The expression level of *CYP81A24* increased prominently by clomazone after clomazone application 0 h and 4 h in R line seedlings (Fig. 8). However, the transcript level of *CYP81A15*, which is very low in both R line and S line seedlings, were not affected by clomazone application (Fig. 8). These results demonstrated that *CYP81A12*, *CYP81A21* and *CYP81A24* play an important role in clomazone resistance in *E. phyllopogon*.

*3.8 Clmazone and 5-keto clomazone metabolic functions of CYP81As*

To evaluate the ability of *CYP81A12*, *CYP81A15*, *CYP81A21*, and *CYP81A24* to metabolize clomazone or 5-keto clomazone, recombinant CYP81A12, CYP81A15, CYP81A21, and CYP81A24 proteins were expressed using *E. coli* expression system that carried the Arabidopsis NADPH-cytochrome P450 reductase gene *ATR1* (Pompon et al., 1996).

For the metabolism assay, clomazone or 5-keto clomazone was added to the cultural media. After 24 hours, the media were analyzed by LC-MS/MS. There are new metabolites peak in transgenic *E.coli* expressing CYP81A12, CYP81A21 and CYP81A24 in clomazone metabolites check and there are also new peaks in transgenic *E.coli* expressing CYP81A15 and CYP81A24 in 5-keto clomazone metabolites check, especially 3 new peaks in transformed CYP81A15, suggesting that CYP81As can metabolize clomazone or 5-keto clomazone directly (Fig. 9). There was no peak detected in the media of *E. coli* expressing other genes, same as in the empty vector control. The reasons for detecting metabolites unsuccessfully maybe due to the expression of the genes is too low to metabolize clomazone or the amount of some metabolites are too small to be detectable.

## 4. Discussion

Clomazone was registered for use in 2002 in California (Tomco et al., 2010), five years after the collection of the resistant line of *E. phyllopogon* from a rice field (Fischer et al., 2000). *E. phyllopogon* exhibits a typical metabolism-based resistance to clomazone, despite the lack of application history. In this study, Arabidopsis transformed with *CYP81A12* or *CYP81A21*, two genes known to play a major role in ALS inhibitor resistance, confer marked resistance to clomazone. Meanwhile, I investigated whether the differential expression *CYP81A12* and *CYP81A21* are involved in difference in clomazone susceptibility between susceptible and multiple-herbicide resistant *E. phyllopogon*. Decreased susceptibility of clomazone were all observed in multiple-herbicide resistant $F_6$ lines, where the two genes are overexpressed, suggesting that the mechanism of clomazone resistance is identical to that of ALS and ACCase herbicide resistance and is caused by the overexpression of *CYP81A12* or *CYP81A21*. The results are consistent with those reported previously (Yasuor, et al., 2008, 2010). Yasuor et al. (2008) suggested the involvement of P450s since P450 inhibitors partially reversed clomazone resistance in the resistant line of *E. phyllopogon*. Another research also reported abundance of oxidized metabolites of clomazone is involved in *E. phyllopogon* resistance to clomazone. Enhanced activity to 5-keto clomazone was also suggested from the fact of lower abundance of 5-keto clomazone in the resistant plants (Yasuor, et al., 2010). Together, upregulation of *CYP81A12* and *CYP81A21* plays an important role in clomazone resistance in *E. phyllopogon*. Although our study suggests that the overexpression of *CYP81A12* and *CY81A21* are very likely involved in clomazone resistance in *E. phyllopogon*, the genetic modification (e.g. knockout) of *E. phyllpogon* has not been established, thus, further research is needed to elucidate the underlying mechanism of clomazone resistance.

Transgenic Arabidopsis response to herbicides revealed that some members of the *CYP81A* subfamily in *E. phyllopogon*, such as *CYP81A15*, *CYP81A18*, and *CYP81A24*, carry a herbicide-metabolizing function as that of *CYP81A12* and *CYP81A21*. These P450s are not involved in the reduced susceptibility to herbicides observed in the resistant line since the expression levels of the corresponding genes were similar between the susceptible and resistant lines of *E. phyllopogon* (Iwakami

et al., 2014a); however, they could be involved in the intrinsic herbicide metabolizing ability of *E. phyllopogon*. *CYP81A*s exclusively exist in Poaceae, which may at least partly explain the relatively lower susceptibility to clomazone and BSM in wild-type *E. phyllopogon* compared with that in wild-type Arabidopsis (Fig. 1, 2) (Iwakami et al., 2014a). Notably, each P450 has substrate preference towards BSM or clomazone. Arabidopsis transformed with *CYP81A12*, *CYP81A18*, or *CYP81A21* showed marked resistance to BSM, whereas Arabidopsis transformed with *CYP81A15* showed high-level resistance to clomazone. Identification of amino acid residues that play an important role in herbicide metabolism may help to better understand the broad substrate specificity observed in the *CYP81A* subfamily.

It is well known that SRSs in P450s act important role in enzyme activities and the catalytic efficiency of P450s can be affected multifariously by polymorphisms (Gotoh, 1992; Schalk and Croteau, 2000). Kahn et al. (2001) reported that a conservative amino acid substitution altered the regiospecificity of CYP94A2 which is a fatty acid hydroxylase from the plant *Vicia sativa*. Amino acid polymorphism which locates in SRS1 between CYP81A15 and other CYP81As was found and then Arabidopsis carrying mutant *CYP81A12*, *CYP81A15* and *CYP81A21* were generated. There is no difference between mutant type and wild type Arabidopsis expressing *CYP81A15* about the susceptibility to BSM and clomazone, while mutant type Arabidopsis expressing *CYP81A12* and *CYP81A21* have increased susceptibility compared with wild type ones, illuminating that the recognition site of BSM and clomazone in *CYP81A15* is not the site in SRS1 where the polymorphism occur, however, this site is very important to metabolize both herbicides for *CYP81A12* and *CYP81A21*. Continuation to investigate the amino acid residues between CYP81A15 and other CYP81As is essential for me to comprehend the involvement of *CYP81A15* in clomazone high-level resistance.

It has been reported that approximately 10% of all P450s are upregulated upon exposure to herbicide (Glombitza et al., 2004; Manabe et al., 2007; Abdeen and Miki, 2009; Das et al., 2010). Bispyribac sodium application could upregulated dozens of P450s in *E. phyllopogon*, but only several genes were identified successfully (Iwakami et al., 2014a). The transcript level of *CYP81A12*, *CYP81A21* and *CYP81A24* were upregulated by clomazone application, which is a part of the stress

response in plants. There will be a number of genes up- or down-regulated after herbicide application, but only a small fraction of the genes are involved in herbicide metabolism and resistance (Delye, 2013). Arabidopsis expressing these three genes exibited resistance to clomazone, further demonstrating their significance in clomazone resistance. Moreover, gene expression in *E. coli* for an *in vitro* clomazone or 5-keto clomazone metabolism assay were conducted: the metabolites of clomazone were detectable in *E.coli* expressing *CYP81A12*, *CYP81A21* and *CYP81A24* and that of 5-keto clomazone were detectable in *E.coli* expressing *CYP81A15* and *CYP81A24*, although the metabolites were unknown, confirming *CYP81A*s can metabolize clomazone directly.

The existence of plant "super P450s" that metabolize various herbicides has long been called in question. Although several herbicide-metabolizing P450s have been identified from multiple plant species, most of them were reported to possess high substrate specificities. For example, Arabidopsis CYP71A12 metabolized only pyrazoxyfen among diverse xenobiotics including 14 herbicides (Hayashi et al., 2007). Similarly, restricted substrate specificity was also observed in CYP81B1 in *Helianthus tuberosus* (Cabello-Hurtado et al., 1998), CYP76Cs in Arabidopsis and CYP72A18 in rice (Imaishi et al., 2007). CYP72A31 can be one of the exceptions as it metabolizes at least two chemical classes of ALS inhibitors (Saika et al., 2014). In contrast to these P450s, members of CYP81A subfamily, at least CYP81A12 and CYP81A21, have broad substrate specificity. CYP81A12 and CYP81A21 metabolize two chemical classes of ALS inhibitors (sulfonylurea and triazolopyrimidine) (Iwakami et al., 2014a), three chemical classes of acetyl-CoA caroboxylase inhibitors (aryloxyphenoxypropionate, cyclohexanedione, and phenylpyrazoline) (Iwakami et al., 2018) and also clomazone. Metabolism-based resistance especially in grasses often accompanies resistance to multiple herbicides as observed in the resistant line of *E. phyllopogon* (Yu and Powles, 2014). CYP81As with herbicide-metabolizing function may also be activated in these resistant plants. Further characterization of CYP81As from other species will provide better understanding of multiple-herbicide-resistance phenomenon in metabolism-based resistance in weeds.

## 5. Summary

Two P450s, *CYP81A12* and *CYP81A21*, which are overexpressed in R population of *E. phyllopogon*, confer clomazone resistance in Arabidopsis, meanwhile, clomazone resistance co-segregated in F6 lines of R line and S line. In addition, the expression level of *CYP81A12* and *CYP81A21* were upregulated by clomazone treatment in R population. All the results strongly suggested that *CYP81A12* and *CYP81A21* are very likely involved in clomazone resistance in R *E. phyllopogon*. Except *CYP81A12* and *CYP81A21*, I found that *CYP81A15* and *CYP81A24* also confer clomazone resistance in Arabidopsis, even though there is no difference in the expression level of them in R line and S line. *CYP81A24* were upregulated by clomazone application in R line, indicating that it may also play an important role in clomazone resistance in R population. CYP81A12, CYP81A15, CYP81A21 and CYP81A24 expressing in *E. coli* could metabolize clomazone or 5-keto clomazone in vitro. These results suggest that P450s are the most important factors that confer clomazone resistance in *E. phyllopogon*, even though every gene confers different resistant level, further explaining clomazone resistance in *E. phyllopogon* is non-target-site resistance. My study provides a foundation to understand the complicated pathways involved in clomazone metabolism in plants.

**Table 1** Resistance and susceptibility to bensulfuron-methyl (BSM) and clomazone in $F_6$ progenies of crosses between susceptible and resistant *Echinochloa phyllopogon*.

| $F_6$ lines | BSM | Clomazone |
|---|---|---|
| SxR-1 | R | R |
| SxR-2 | S | S |
| SxR-3 | R | R |
| SxR-4 | S | S |
| SxR-5 | S | S |
| SXR-6 | S | S |
| SxR-7 | S | S |
| SxR-8 | R | R |
| SxR-9 | S | S |
| SxR-10 | S | S |
| SxR-11 | S | S |
| SxR-12 | R | R |
| SxR-14 | S | S |
| SxR-15 | R | R |
| SxR-16 | S | S |
| SxR-17 | R | R |
| SxR-18 | R | R |
| SxR-19 | S | S |
| SxR-20 | S | S |
| SxR-21 | S | S |
| SxR-22 | R | R |
| SxR-23 | S | S |
| SxR-24 | R | R |
| SxR-26 | R | R |
| SxR-27 | S | S |
| SxR-29 | S | S |
| SxR-30 | R | R |
| SxR-31 | R | R |
| SxR-32 | R | R |
| SxR-34 | R/S | R/S |
| SxR-35 | R | R |
| SxR-36 | R | R |
| SxR-37 | S | S |
| SxR-39 | S | S |
| SxR-40 | S | S |
| SxR-41 | S | S |
| SxR-42 | S | S |
| SxR-43 | S | S |
| SxR-45 | S | S |
| SxR-46 | S | S |
| SxR-47 | S | S |
| SxR-49 | R | R |
| SxR-50 | S | S |
| SxR-51 | R | R |
| SxR-53 | R | R |
| SxR-54 | S | S |
| SxR-55 | S | S |
| SxR-56 | R | R |
| SxR-59 | S | S |
| SxR-61 | S | S |

Note: R, resistant; S, Sensitive; R/S, segregated.

**Table 2.** Primers used in this study.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| **Full-length amplification for Arabidopsis transformation** | | |
| *CYP81A14* | 5'-caccTATTGTAGGATTGCGGCGGTA-3' | 5'-ACGTGTTCAGCTTCACCTCATTC-3' |
| *CYP81A15* | 5'-caccAGAAAGACAGGTCCTAAACC-3' | 5'-AAAGCTATAATGTCGGTTCCCGTTCC-3' |
| *CYP81A18* | 5'-caccGCGCAGAAAGACACAG-3' | 5'-AGAATATGGTCATCGAGCAGGAAC-3' |
| *CYP81A23* | 5'-caccGATAATTGCAATCCGCCGATCA-3' | 5'-GTACAAACAAGTGAAGGTAGTGAGAAAC-3' |
| *CYP81A24* | 5'-caccAGCGAGATCAATCAGAAAC-3' | 5'-ATATGTGATGATAAAATAAGCCACAAAC-3' |
| *CYP81A26* | 5'-caccGCCCAAAAAACATGGAG-3' | 5'-AACACATTGCATCCATCTACG-3' |
| **Real-time PCR in *Arabidopsis*** | | |
| *CYP81A14* | 5'-AAAATATCATTTGTTAGCAGTTCTGG-3' | 5'-CAAACATTTTGTAGGATGTTTCAGC-3' |
| *CYP81A15* | 5'-TTCAGGAAATCTAGGATGGAG-3' | 5'-GACCGGGTAAATGCAAAGAA-3' |
| *CYP81A18* | 5'-GGTGAAGTTGTGATATTTTATAGGAGA-3' | 5'-AGAATATGGTCATCGAGCAGGAAC-3' |
| *CYP81A22* | 5'-GCTCATGTCATGTCTGGTACTCTG-3' | 5'-ACACGAACTGACGAACTAAACGTC-3' |
| *CYP81A23* | 5'-TCACGTACTTCTCCCCAACC-3' | 5'-TGCATGCCTACTGCTATGTG-3' |
| *CYP81A24* | 5'-TTCAGGAGCTCTGATGGAAG-3' | 5'-CGAATGGTCAAACGTTACGAG-3' |
| *CYP81A26* | 5'-AAGGGGTCGGGATTACCCTA-3' | 5'-CAAATCCTTCTTCTTCATATTCACAG-3' |
| *GAPDH* | 5'-TTGGTGACAACAGGTCAAGCA-3' | 5'-AAACTTGTCGCTCAATGCAATC-3' |
| **Real-time PCR in *E. phyllopogon*** | | |
| *CYP81A12* | 5'-GGTAGCTGATAGCGTGTGCTG-3' | 5'-TTCATACAAACCCCTCTACACACA-3' |
| *CYP81A15* | 5'-TTCAGGAAATCTAGGATGGAG-3' | 5'-GACCGGGTAAATGCAAAGAA-3' |
| *CYP81A21* | 5'-ATGCAGGAAAGCTTCGTCTATAGATT-3' | 5'-CTTTAATCGTTCATACAAACCCAGT-3' |
| *CYP81A24* | 5'-TTCAGGAGCTCTGATGGAAG-3' | 5'-CGAATGGTCAAACGTTACGAG-3' |
| **Vector construction for *E.coli* expression** | | |
| GbSc-81A12syn-Fw | 5'-cccgggtaccaaaaaaATGGATAAGGCCTACGTGGCCGTC-3' | |
| GbSc-81A12syn-Rw | 5'-atcccccgcggaattcTCAGAGCTCCTGAAGAACATCATG-3' | |
| GbSc-81A15syn-Fw | 5'-cccgggtaccaaaaaaATGGAAAGATTCTACTACGTCGCC-3' | |
| GbSc-81A15syn-Rw | 5'-atcccccgcggaattcCTAGATTTCCTGAAGAAGAGCGG-3' | |
| GbSc-81A21syn-Fw | 5'-cccgggtaccaaaaaaATGGATAAGGCCTACGTGGCCGTC-3' | |
| GbSc-81A21syn-Rw | 5'-atcccccgcggaattcTCAGAGCTCCTGAAGAACATCATG-3' | |
| GbSc-81A24syn-Fw | 5'-cccgggtaccaaaaaaATGGATAAGGCCTACATCGCCGTC-3' | |
| GbSc-81A24syn-Rw | 5'-atcccccgcggaattcTCAGAGCTCCTGAAGGACACGGCG-3' | |

Note: Lower case represents nucleotides for directional cloning with pENTR-D-TOPO.

**Table 3.** Plasmids and bacterial strains used in this study

| Plasmid or strain | Relevant characteristic(s) | Reference or source |
|---|---|---|
| Plasmids | | |
| pET28-2C3-81A12 | pET28-a(+) carrying 2C3-81A12 | This study |
| pET28-2C3-81A15 | pET28-a(+) carrying 2C3-81A15 | This study |
| pET28-2C3-81A18 | pET28-a(+) carrying 2C3-81A18 | This study |
| pET28-2C3-81A21 | pET28-a(+) carrying 2C3-81A21 | This study |
| pET28-2C3-81A24 | pET28-a(+) carrying 2C3-81A24 | This study |
| pCDF-ATR2-hemA | pCDF-1b carrying transmembrane region truncated ATR2 and hemA | This study |
| pET28-a(+) | | Merck Millipore |
| pGro7 | groES and groEL expression plasmid | Takara |
| | | |
| Strains | | |
| E. coli C41(DE3) | | Lucigen |
| C41-pET28 | C41(DE3) carrying pET28-a(+), pCDF-ATR2-hemA, and pGro7 | This study |
| C41-2C3-81A12 | C41(DE3) carrying pET28-2C3-81A12, pCDF-ATR2-hemA, and pGro7 | This study |
| C41-2C3-81A15 | C41(DE3) carrying pET28-2C3-81A15, pCDF-ATR2-hemA, and pGro7 | This study |
| C41-2C3-81A18 | C41(DE3) carrying pET28-2C3-81A18, pCDF-ATR2-hemA, and pGro7 | This study |
| C41-2C3-81A21 | C41(DE3) carrying pET28-2C3-81A21, pCDF-ATR2-hemA, and pGro7 | This study |
| C41-2C3-81A24 | C41(DE3) carrying pET28-2C3-81A24, pCDF-ATR2-hemA, and pGro7 | This study |

**Table 4.** Relative expression of *CYP81A* genes of *Echinochloa phyllopogon* in Arabidopsis transformed with each gene.

| *CYP81A14* | | *CYP81A15* | | *CYP81A18* | | *CYP81A22* | | *CYP81A23* | | *CYP81A24* | | *CYP81A26* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Line | Expression | Line | Expression | Line | Expression | Line | Expression | Line | Expression | Line | Expression | Line | Expression |
| 81A14#2-1 | 269.2±0.1 | 81A15#12-1 | 1.7±0.6 | 81A18#9-6 | 56.1±5.6 | 81A22#6-3 | 5.0±2.5 | 81A23#41-3 | 1.3±0.003 | 81A24#1-1 | 2.6±0.08 | 81A26#52-3 | 449.2±1.0 |
| 81A14#5-2 | 8.3±0.005 | 81A15#13-2 | 1.8±0.3 | 81A18#11-5 | 21.3±1.6 | 81A22#11-4 | 17.8±1.5 | 81A23#46-2 | 1.0±0.006 | 81A24#15-1 | 1.0±0.1 | 81A26#54-3 | 2.7±0.005 |
| 81A14#45-4 | 1.0±0.0004 | 81A15#14-8 | 1.0±0.05 | 81A18#18-6 | 1.0±0.03 | 81A22#12-6 | 14.0±9.1 | 81A23#47-4 | 2.5±0.03 | 1A24#21-5 | 1.5±0.2 | 81A26#56-4 | 1.0±0.0004 |
| 81A14#52-1 | 1.7±0.001 | 81A15#15-6 | 10.3±1.4 | 81A18#28-6 | 2.3±0.3 | 81A22#14-4 | 15.7±8.4 | 81A23#48-4 | 4.8±0.03 | | | | |
| | | | | | | 81A22#15-6 | 1.0±0.3 | 81A23#49-2 | 44.9±0.5 | | | | |
| | | | | | | 81A22#17-3 | 73.6±13.0 | | | | | | |
| | | | | | | 81A22#20-5 | 71.8±10.8 | | | | | | |

Note: Arabidopsis lines with highlight were used in this study.

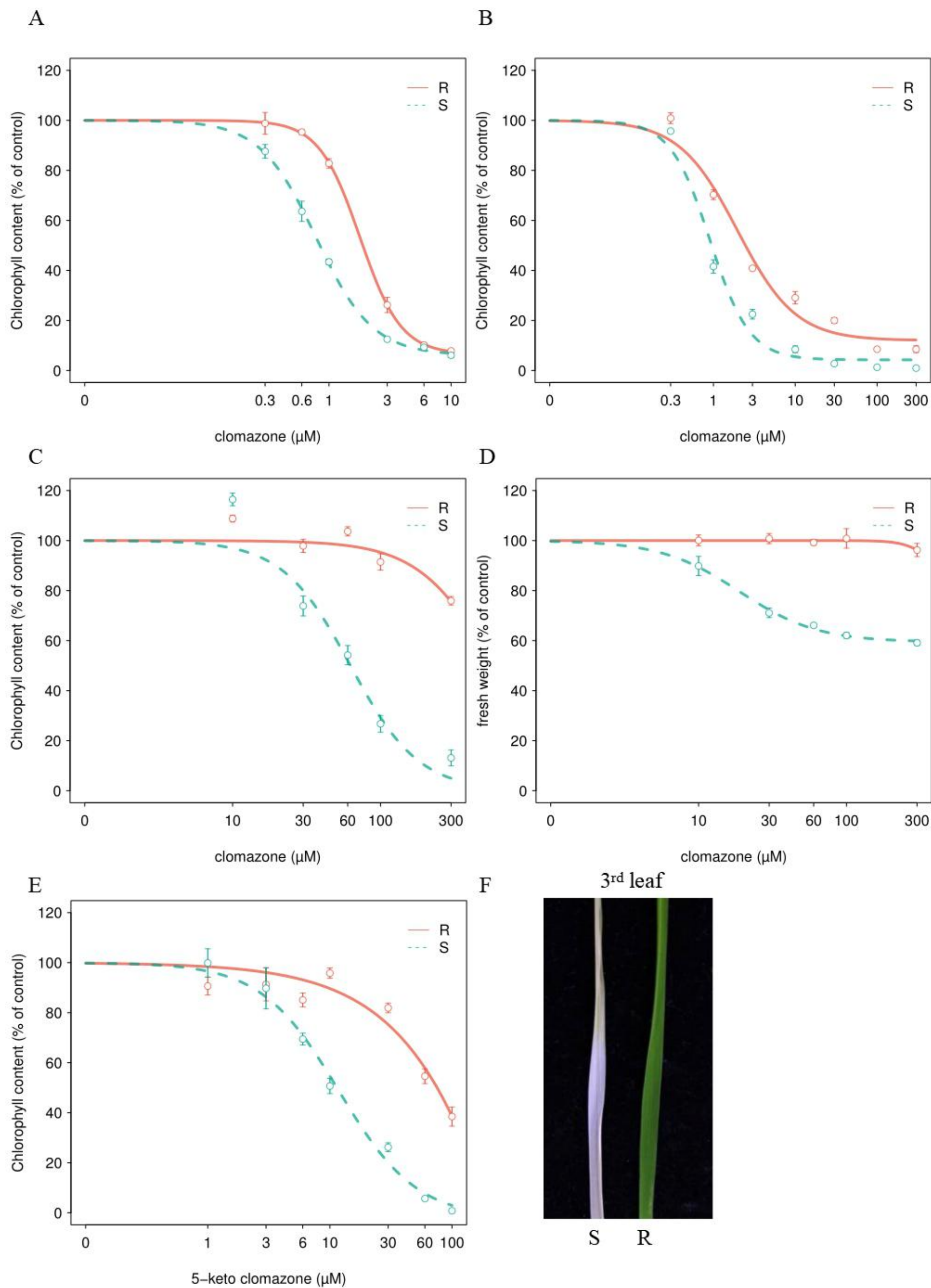


**Fig. 1.** Susceptibility to clomazone and 5-keto clomazone in resistant (R) and susceptible (S) lines of *Echinochloa phyllopogon*. A, Dose response curve of R and S lines to root-treated clomazone in the germinated seedlings. Susceptibility was evaluated by the relative chlorophyll content of the whole seedlings. Bars represent standard deviation (n = 4). B, Dose response curve of R and S lines to root-treated clomazone in the 2-leaf stage seedlings. Susceptibility was evaluated by the relative chlorophyll content of the 3rd leaf. Bars represent standard deviation (n = 4). C, Dose

response curve of R and S lines to leaf-treated clomazone in the 2-leaf stage seedlings. Susceptibility was evaluated by the relative chlorophyll content of the 3rd leaf. Bars represent standard deviation (n = 4). D, Dose response curve of R and S lines to leaf-treated clomazone in the 2-leaf stage seedlings. Susceptibilities were evaluated by relative fresh weight of whole seedlings. Bars represent standard deviation (n = 4). E, Dose response curve of R and S lines to leaf-treated 5-keto clomazone in the 2-leaf stage seedlings. Susceptibility was evaluated by the relative chlorophyll content of the 3rd leaf. Bars represent standard deviation (n = 4). F, The third leaf of R and S lines at 6 d after clomazone application (100 μM).

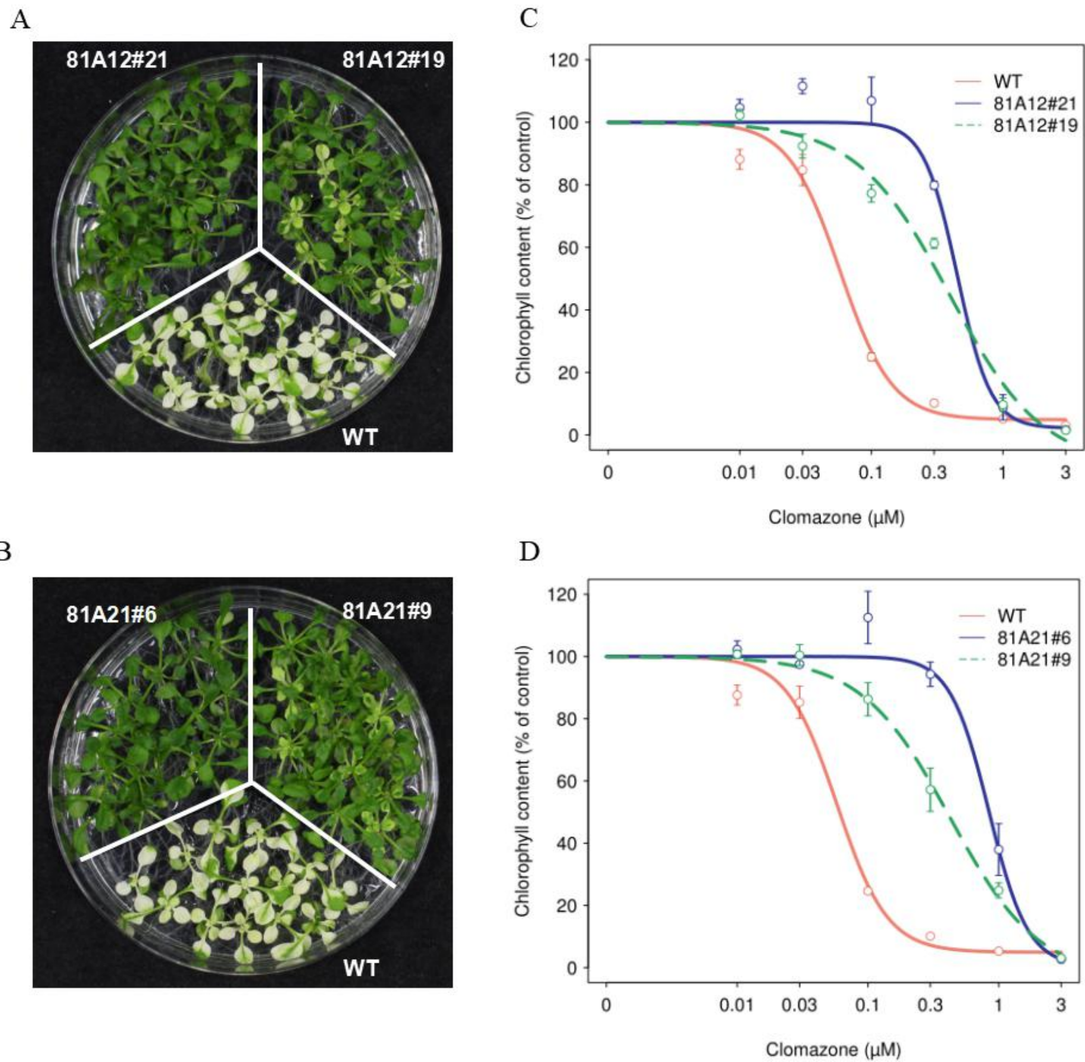


**Fig. 2.** Susceptibility to clomazone in Arabidopsis transformed with *CYP81A12* or *CYP81A21*. A and B, Seedlings grown for 14 d on Murashige and Skoog (MS) medium with 0.1 μM clomazone. C and D, Clomazone susceptibility of independent transgenic lines on MS medium containing clomazone. Susceptibility was evaluated by the relative chlorophyll content. Bars represent standard deviation (n = 4).

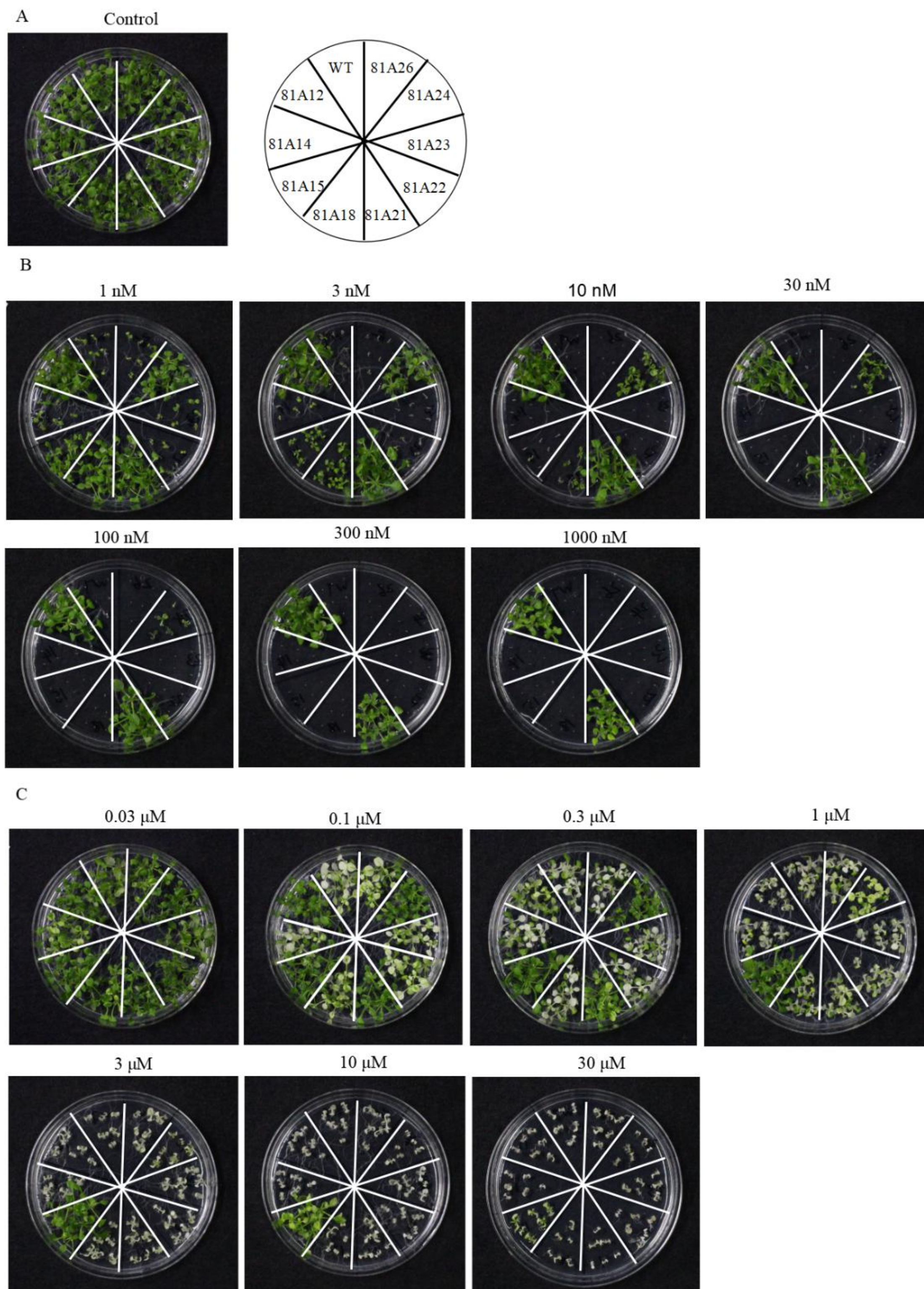


**Fig. 3.** Susceptibility to clomazone and bensulfuron-methyl (BSM) in Arabidopsis transformed with *CYP81A*s of *Echinochloa phyllopogon*. Seedlings grew for 14 d on Murashige and Skoog (MS) medium containing BSM (B) or clomazone (C). WT,

wild-type; 81A12#21-10, *CYP81A12* line; 81A14#2-1, *CYP81A14* line; 81A15#15-6, *CYP81A15* line; 81A18#9-6, *CYP81A18* line; 81A21#6-1, *CYP81A21* line; 81A22#17-3, *CYP81A22* line; 81A23#9-2, *CYP81A23* line; 81A24#1-1, *CYP81A24* line; 81A26#52-3, *CYP81A26* line. A, Control and plate arrangement.

| | 112 | 116 | 121 | 122 | 123 | 225 | 310 | 311 | 318 |
|---|---|---|---|---|---|---|---|---|---|
| CYP81A12 | Q | S | S | L | S | I | M | S | G |
| CYP81A21_R | Q | S | S | L | S | I | M | S | G |
| CYP81A21_S | Q | S | S | L | S | I | M | S | G |
| CYP81A22_R | M | A | M | L | S | V | M | A | G |
| CYP81A22_S | M | A | M | L | F | V | M | A | G |
| CYP81A24 | M | S | M | L | S | V | M | A | G |
| CYP81A23 | Q | L | A | L | T | V | M | A | G |
| CYP81A26_R | L | S | T | L | P | I | M | A | T |
| CYP81A26_S | L | S | T | L | P | I | M | A | T |
| CYP81A15 | L | T | M | P | T | Q | N | A | S |
| CYP81A14 | V | S | V | L | T | I | S | A | G |
| CYP81A18 | V | S | V | L | T | I | N | A | G |

**Fig. 4.** Alignment of partial protein sequences of the CYP81A subfamily of a resistant (R) line and a susceptible (S) line. The residue 122 of all the CYP81As is Leu except CYP81A15 which is replaced by Pro.

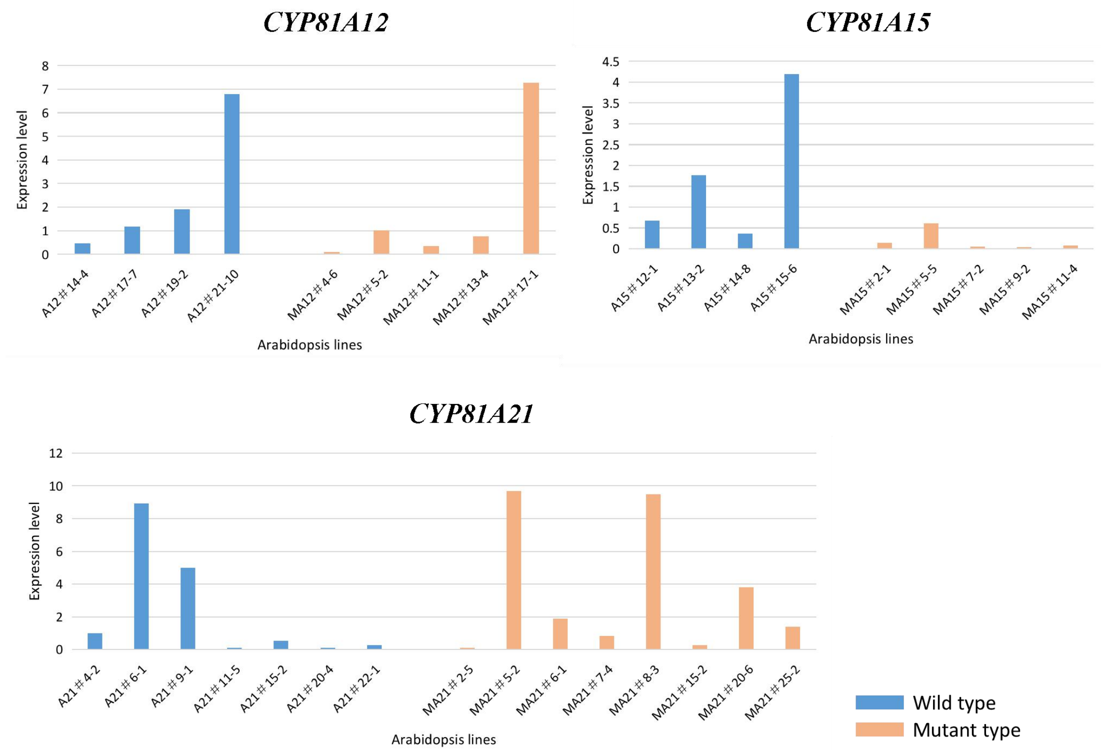


**Fig. 5.** Transcript levels of *CYP81A12*, *CYP81A15* and *CYP81A21* in wild type and mutant type Arabidopsis. Transformed Arabidopsis lines shown are T3 homozygous lines. Seedlings grew for 14 d on Murashige and Skoog (MS) medium and then were collected to extract RNA. mRNA levels of the transgene in independent transgenic lines. The transcript levels of *CYP81A12*, *CYP81A15* and *CYP81A21* were quantified by real-time RT-PCR using *GAPDH* as an internal control gene. Blue column represents Arabidopsis carrying wild type *CYP81A12*, *CYP81A15* and *CYP81A21*; Orange column represents Arabidopsis carrying mutant type *CYP81A12*, *CYP81A15* and *CYP81A21*.

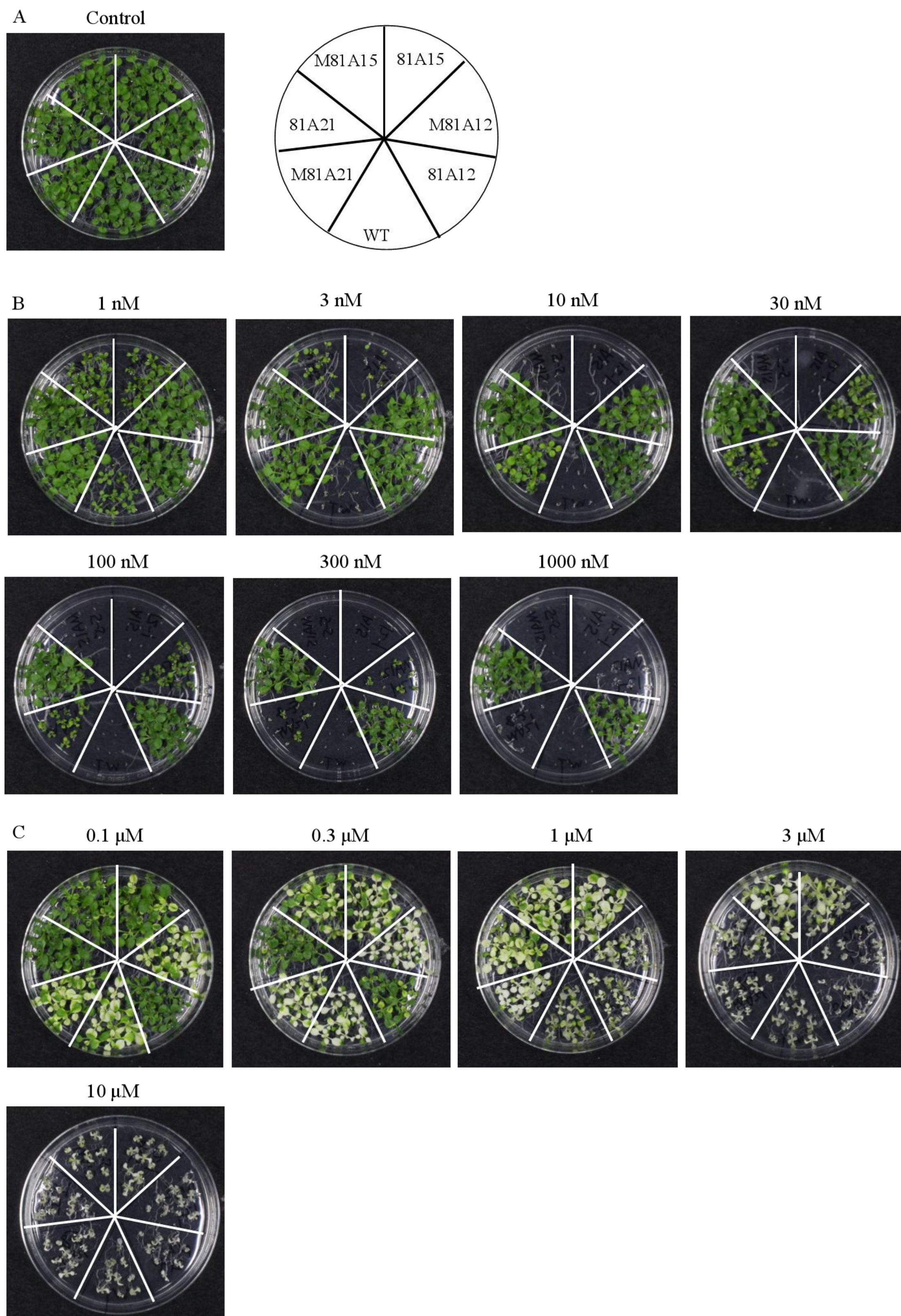


**Fig. 6.** Susceptibility to bensulfuron-methyl (BSM) and clomazone in Arabidopsis transformed with mutant *CYP81A*s of *Echinochloa phyllopogon*. Seedlings grew for 14 d on Murashige and Skoog (MS) medium containing BSM or clomazone. WT,

wild-type; 81A12#21-10, *CYP81A12* line; M81A12#17-1, mutant *CYP81A12* line; 81A15#12-1, *CYP81A15* line; M81A15#5-5, mutant *CYP81A15* line; 81A21#6-1, *CYP81A21* line; M81A21#8-3, mutant *CYP81A21* line. A, Control and plate arrangement.

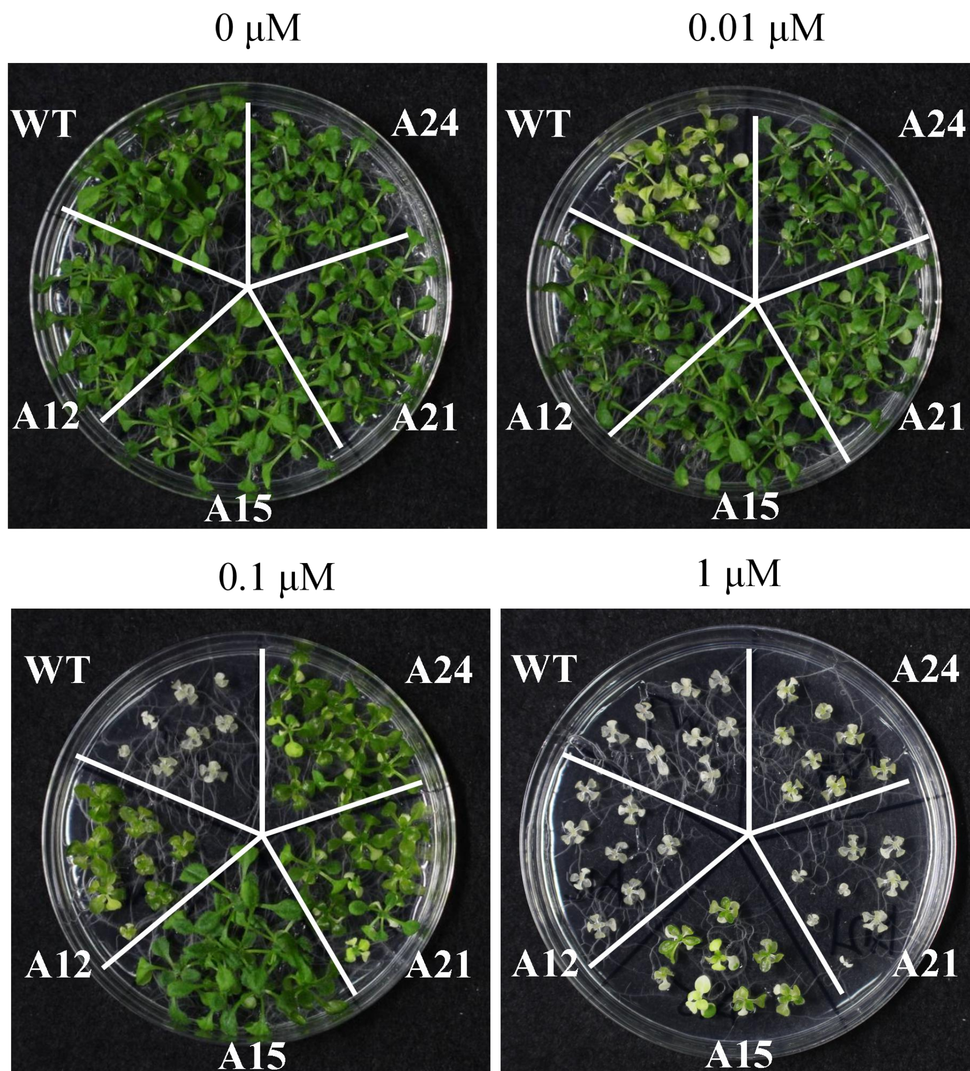


**Fig. 7.** Susceptibility to 5-keto clomazone in Arabidopsis transformed with *CYP81A*s of *Echinochloa phyllopogon*. Seedlings were grown for 14 d on Murashige and Skoog (MS) medium containing 5-keto clomazone. WT, wild-type; 81A12#10-1, *CYP81A12* line; 81A15#15-6, *CYP81A15* line; 81A21#6-1, *CYP81A21* line; 81A24#1-1, *CYP81A24* line.

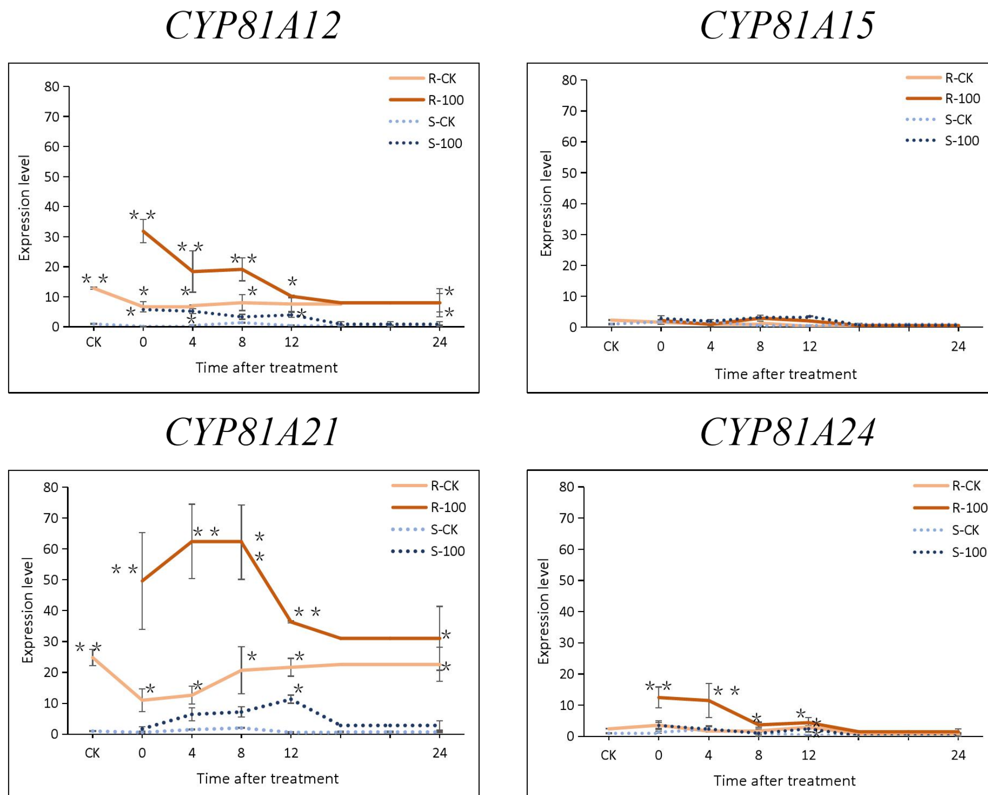


**Fig. 8.** Comparison of transcript levels of P450 genes in the leaves of R and S *Echinochloa phyllopogon* between treated (100 μM) and non-treated clomazone. clomazone (100 μM) was applied to leaves when seedlings grew to 2-leaf stage; before clomazone application, leaf samples were collected recording as CK; control of R and S seedlings were applied with distilled water. After clomazone application 0 h, 4 h, 8 h, 12 h and 24 h, leaf samples were collected, respectively. Transcript levels were examined by real-time RT-PCR using *EIF4B* as internal control genes. Transcript abundance was normalized to the level in the shoot of untreated S line. Data shown are means ± SD of three biological replicates (Student's t test, ＊ $P<0.05$, ＊＊ $P<0.01$).

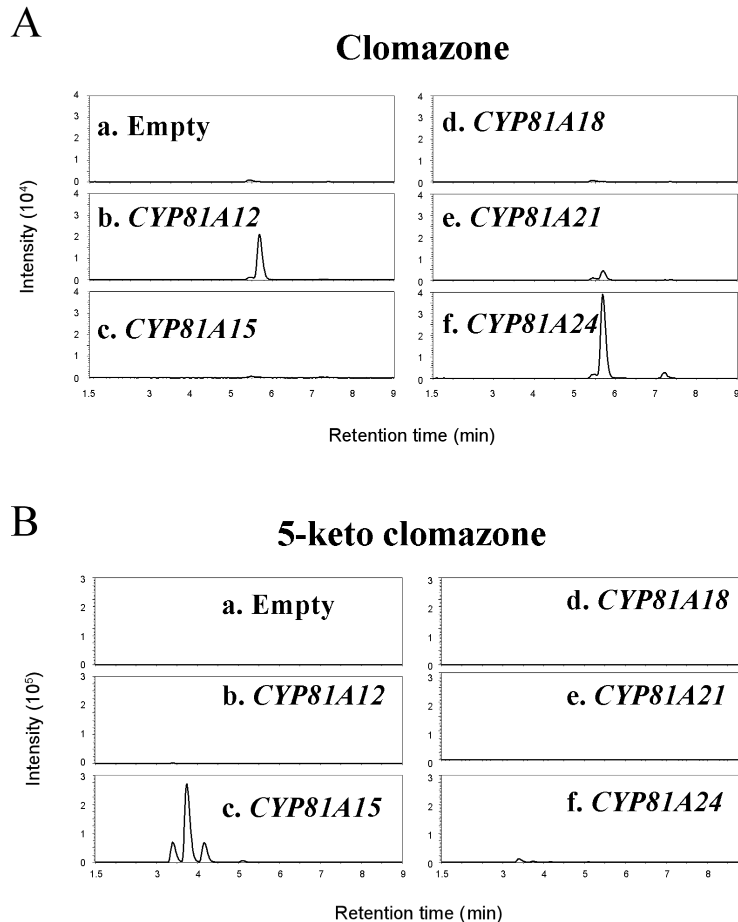


**Fig. 9.** Clomazone and 5-keto clomazone catalyzed by *CYP81A*s of *Echinochloa phyllopogon*. A, LC-MS/MS analyses of a clomazone metabolite formed in *E. coli* expressing *CYP81A*s. a, Chromatogram of *E. coli* harboring the empty vector (pET28) as a control. b, Chromatogram of *E. coli* expressing an R allele of *CYP81A12*. A new peak was detected. c, Chromatogram of *E. coli* expressing an R allele of *CYP81A15*. d. Chromatogram of *E. coli* expressing an R allele of *CYP81A18*. e. Chromatogram of *E. coli* expressing an R allele of *CYP81A21*. A new peak was detected. f. Chromatogram of *E. coli* expressing an R allele of *CYP81A24*. A new peak was detected. B, LC-MS/MS analyses of 5-keto clomazone metabolite formed in *E. coli* expressing *CYP81A*s. a, Chromatogram of *E. coli* harboring the empty vector (pET28) as a control. b, Chromatogram of *E. coli* expressing an R allele of *CYP81A12*. c, Chromatogram of *E. coli* expressing an R allele of *CYP81A15*. Three new peaks were detected. d. Chromatogram of *E. coli* expressing an R allele of *CYP81A18*. e. Chromatogram of *E. coli* expressing an R allele of *CYP81A21*. f. Chromatogram of *E. coli* expressing an R allele of *CYP81A24*. A new peak was detected.

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